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PROCEEDINGS
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NATIONAL ACADEMY OF SCIENCES

THE CONFERENCE ON BIOELECTRIC POTENTIALS

By Duncan A. MacInnes

Rockefeller Institute for Medical Research

Communicated July 8, 1949

The first of a series of conferences on various rapidly developing subjects in science, under the Auspices of the National Academy of Sciences, was that on Bioelectric Potentials. It was held at the Rockefeller Institute for Medical Research in New York City, on December 14, 1946, in honor of Dr. W. J. V. Osterhout.

The discussion during the two-day session centered around four papers which were:

"Some Bioelectrical Problems," by W. J. V. Osterhout.
"The Source of Bioelectric Potentials in Large Plant Cells," by Lawrence Blinks.
"Physical Aspects of Bioelectric Phenomena," by Kenneth S. Cole.
"Nerve Action," by R. Lorente de Nó.

The first three of these papers, revised in the light of the discussions, follow in this number of the Proceedings.

The participants in the conference were: L. R. Blinks, Detlev W. Bronk, Kenneth S. Cole, Richard T. Cox, P. Debye, Joseph Erlanger, Teh-Pei Feng, W. O. Fenn, R. H. Fuoss, Herbert S. Gasser, H. S. Harned, R. Höber, J. G. Kirkwood, Irving Langmuir, David P. C. Lloyd, L. G. Longsworth, D. A. MacInnes, R. Lorente de Nó, L. Michaelis, John von Neumann, Lars Onsager, W. J. V. Osterhout, Alexandre Rothen, Francis O. Schmitt and Theodore Shedlovsky.

The other conferences that have been held so far are as follows:

Subject	*Chairman*	*Date*
Foundations of Quantum Mechanics	K. K. Darrow	June 2–4, 1947
Theoretical Physics	J. R. Oppenheimer	March 30 to April 2, 1948
Low Temperature Physics	J. C. Slater	May 31, June 1–2, 1948
The Gene	B. P. Kaufmann	May 31, June 1–2, 1949
The Ultracentrifuge	D. A. MacInnes	June 12–14, 1949

These conferences were held at the Rams Head Inn, Shelter Island, L. I., N. Y., except the second which took place at Pocono Manor, Pa.

The conferences have been organized so as to bring together active workers in order to consider current research problems in a selected field of science. Formal presentation of papers has been avoided, the emphasis being on leisurely and informal discussion. The number of participants in each conference has been limited to 25. After the first conference, which was held in New York City, the remaining ones have been held at country inns.

It has been found that much of value may be accomplished in two or three days of close association by key workers, who are frequently widely scattered geographically. Concentration on a given topic, the absence of distracting interests and the opportunity of free intimate discussion which is made possible by a small group, are particularly helpful.

These conferences have been made possible by the advice and encouragement of Past President Jewett and President Richards, both of whom assigned Academy funds for the expenses involved.

SOME BIOELECTRICAL PROBLEMS

By W. J. V. Osterhout

The Laboratories of the Rockefeller Institute for Medical Research

Communicated July 8, 1949

What would Galvani say if he could join us today? Perhaps the conversation would run as follows.

"In 1791 I published some observations on frogs' legs. I noticed that in contact with copper and iron they twitched vigorously. Can you tell me why this happens?"

"We fear that we cannot give you a satisfactory answer. In spite of much study the cause is not clear."

"It is a long time," he answers, "since the first observations were made."

"It is true, but we need more help from the physicists and physical chemists to solve this problem. That is the reason for this conference."

"Well," he says finally "how far have you got?"

A clear and simple answer is not easy to give. Much progress has been made but much remains obscure. For the purpose of our conference it may be best to consider some fundamental questions which can be so stated as to appeal to the physicist and to the physical chemist.

If I attempt a brief statement of this kind as requested by our chairman I must ask your indulgence.

The behavior which astonished Galvani is due to disturbances in the electrical potentials which exist at the surfaces of living cells. We can measure these potentials and the rate at which these disturbances travel along the cell and we can subject our observations to mathematical treatment. We have made some progress in analyzing the mechanism by which an electrical disturbance is produced and propagated. In some cases the potentials appear to be due chiefly to diffusion potentials set up by potassium compounds against the non-aqueous surface films of the protoplasm. In such cases we can measure the mobilities of the ions in the non-aqueous films and show that they account for the observed potentials. These mobilities are usually very different from those found in water.

If a spot with normal resting potential is connected through a galvanometer to another spot on the same cell where the resting potential has been reduced to zero (by applying KCl) we find in some cases that a current of several microamperes flows from the normal to the treated spot and this may persist for several hours. It would appear that phase boundary potentials as ordinarily understood could not account for such currents.

To illustrate some of the methods employed in such studies we may consider experiments with a plant cell, *Nitella*, which acts somewhat like muscle and nerve but is in some respects easier to study.

The protoplasm of the *Nitella* cell (Fig. 1) forms a thin layer (not over 15 microns thick) surrounding a large central vacuole (about 500 microns in diameter) filled with sap; outside the protoplasm is a cellulose wall which is so permeable that it does not affect the potentials (Fig. 1). We can obtain sap from the interior of such cells without external contamination and we can follow changes in its composition by expressing and analyzing it.

Surface Films.—The surfaces (both the outer surface and the inner surface surrounding the vacuole) of the protoplasm are completely covered with a film of oily or non-aqueous material too thin to be visible under the microscope and hence less than half a wave-length of visible light in thickness. We do not know whether these films are solid or liquid or an emulsion but they act like liquid in obeying the laws of surface tension.

Although protoplasm is largely composed of water it rounds up in contact with water like an oily liquid because of the non-aqueous film covering its surface. This is easily seen if the cellulose wall is pierced with a needle so that the protoplasm protrudes into water or if the protoplasm is

withdrawn from the cellulose wall by a plasmolyzing solution which fills the space between the protoplasm and the cellulose wall. The inner protoplasmic surface acts in similar fashion as is evident when it is rapidly deformed by protoplasmic motion.

It is natural to assume that such films are formed by substances which lower the surface tension of the protoplasm and which therefore accumulate in the surface since, if they diffuse into it, energy is required to get them out. We might therefore expect the inner and outer protoplasmic surfaces to be similar. This is approximately true of *Nitella* for if we place sap outside the cell and form the chain

Sap | Protoplasm | Sap

we find an e. m. f. of only 16 mv.[1]

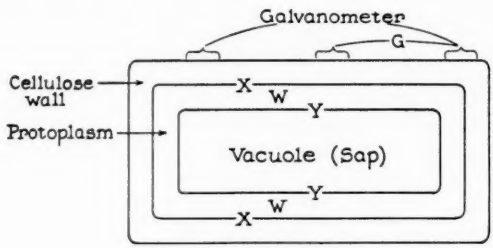

FIGURE 1

Diagram to represent the structure of a *Nitella* cell. The protoplasm consists of an aqueous layer, *W*, with a film of non-aqueous material, *X*, at the outer surface and a corresponding film, *Y*, at the inner surface. The vacuole is filled with sap containing about 0.05 *M* KCl and 0.05 *M* NaCl with a very small amount of other electrolytes and of organic matter. (The relative diameter of the vacuole is much greater than is here represented.)

These films determine what substances enter and leave the cell and thus control metabolism. They furnish the best test of life which we possess since if they are irreversibly destroyed life ceases at once. This is indicated by the entrance of such dyes as trypan blue or acid fuchsin which do not enter the cell as long as the surface films are intact and which thus provide a useful test of death. We may say that the cell is dead when the protoplasm becomes completely and irreversibly permeable as happens when the surface film is destroyed.

The destruction of the surface film may be brought about by agents which act on the film or on the substrate of protoplasm on which the film rests.

The bioelectrical potentials are set up at these films. Since the study of

such films involves surface chemistry and physics which are little understood it is highly desirable to find suitable models for study.

Attempts have been made to produce such films between two aqueous solutions to imitate the conditions in the living cell but so far the results have not been satisfactory. One method is to fill a tube with aqueous solution and place it upright in a non-aqueous liquid and then allow a drop of aqueous solution hanging from another tube to descend slowly until it compresses the non-aqueous liquid between the two aqueous solutions. In this way very thin non-aqueous films can be obtained and electrical measurements across them can be carried out, but the films soon disrupt. Films have also been made by blowing bubbles with non-aqueous materials but they do not last long.

Resting Potentials.—By means of its surface films the cell can act very much like a potassium electrode. To see this we need only connect a galvanometer between a spot on the cell where the potential is kept constant to another spot which is in contact with an aqueous solution of KCl. As the concentration of KCl at the latter spot changes while that at the other spot remains constant the P.D. changes in such fashion as to obey the equation for diffusion potential which for 25°C. may be written

$$\text{P.D.} = 59 \frac{u_K - v_{Cl}}{u_K + v_{Cl}} \log \frac{a_1}{a_2}$$

Here u_K and v_{Cl} are the mobilities of K^+ and of Cl^- in the outer non-aqueous protoplasmic surface film, X; a_1 and a_2 are the activities[2] in X of K^+ (taken as equal to those of Cl^-) when two solutions are applied to X in succession so as to set up a diffusion potential in X. We find that u_K may be 73 times v_{Cl} which shows how much the surface of the protoplasm differs from water in which $u_K \div v_{Cl} = 0.96$.

To carry out such measurements we allow the cell to remain for an hour or more in contact with 0.001 M KCl. We assume that the outermost part of X has reached approximate equilibrium with the external solution so that $a_2 = 0.001\ (S)$ where S is the activity partition coefficient of K^+ (taken as equal to that of Cl^-).[3] We then replace 0.001 M KCl by 0.01 M KCl and assume that a diffusion gradient between the two activities of K^+ is set up in X and that $a_1 = 0.01\ (S)$. Since the value of S remains constant[4] as long as the properties of the two phases (X and the external solution) remain constant a tenfold change in the activity of K^+ in the external solution means a tenfold change in the activity of K^+ in X.

When we set up models and use various non-aqueous substances in place of X we find that a tenfold change of activity of KCl in the aqueous solution gives different values for the P.D. depending on the nature of the non-aqueous phase. This must be due to differences in u_K and v_{Cl} if the activity partition coefficient S is constant.

The situation may be more complicated if other ions in the surface play an important part, but for our present purpose they may be left out of consideration since as a rule the effect in *Nitella* is chiefly due to KCl.

It may be asked whether the surface behaves like a system of charged pores such as exist in a collodion membrane. This is evidently not the case for in *Nitella* we find that under some conditions the order of mobilities in X is $u_K > v_{OH} > u_{Na}$ and in some other plants it is $u_K > v_{Cl} > u_{Na}$. In a system of charged pores all cations are faster than all anions or vice versa.

By means of its surface films the protoplasm is able to distinguish clearly between NaCl and KCl. When 0.01 M KCl in contact with *Nitella* is replaced by 0.01 M NaCl the outwardly directed potential is increased by about 80 mv. This has been called the potassium effect.

Some substance (or group of substances) in the protoplasmic surface must be responsible for this and a number of materials have been examined from this point of view. The most interesting so far tested is nitrobenzene. When two tubes filled with aqueous solutions dip into nitrobenzene, one containing 0.1 M KCl and the other 0.1 M NaCl, we find on connecting them through a galvanometer that 0.1 M NaCl is positive to 0.1 M KCl just as in *Nitella*. In the living cell the value is about 80 mv. and in nitrobenzene about 67 mv.

It would be very interesting to know how nitrobenzene produces this result. We hope that some physical chemist will look into this.

The non-aqueous films at the surfaces of the protoplasm are easily altered. Leaching *Nitella* with distilled water reduces the relative mobility of K^+ in the outer protoplasmic surface so that $u_K \div u_{Na}$ falls from 33 to 1 and $u_K \div v_{Cl}$ falls from 73 to 2.2. Hence the cell is no longer able to act like a potassium electrode or to distinguish electrically between K^+ and Na^+. At the same time it loses its power to respond to an electrical stimulus. Evidently a substance or group of substances is removed from the surface films; it seems possible that such substances are found in blood for if we apply dilute blood to the leached cell for a few seconds we restore its ability to distinguish between K^+ and Na^+ and to respond to electrical stimulation.

This important substance exists in very minute amounts and we need the help of the chemist to study it. A beginning has been made by taking water in which *Nitella* has been standing and shaking it with chloroform. A precipitate then separates out at the surfaces of the drops of chloroform. When this is redissolved in water and applied to the cells it restores them to their normal state. But the amount of substance thus obtained is very small and we need better methods.

Interesting alterations in the surface films are produced by guaiacol. In *Nitella* it alters mobilities and in *Valonia* it changes the order $u_K > v_{Cl} > u_{Na}$ to $u_{Na} > v_{Cl} > u_K$.

It is evident that changes in the surface films due to metabolism might alter mobilities. A variety of changes may occur under normal conditions in the surface films. This is not surprising for any substances produced in the cell which have the power to lower surface tension become trapped if they enter the surface since energy is required to get them out. Partition coefficients may also be altered so that without any change in the concentration of NaCl or of KCl in the external solution their relative proportions in the non-aqueous surface film may change and thus affect the potential.

Since the protoplasmic surface acts like guaiacol in some respects a study of partition coefficients in guaiacol has been made by Shedlovsky and Uhlig which is very helpful in dealing with the living cell.

The partition coefficients in *Nitella* may be calculated as follows. Putting for convenience $v_{Cl} = 1$ and ascertaining the relative values of u_K and u_{Na}, as already described (p. 550) we can predict the value to be expected in a given mixture of NaCl and KCl. Any divergence from the predicted value is then attributed to differences in the partition coefficients and these are evaluated. They are then used to predict values expected in other mixtures and if these prove to be approximately correct we assume that we have ascertained the approximate values of the partition coefficients. When we apply this same method to guaiacol in a U-tube used in place of *Nitella*, we get approximately correct values.

What determines the magnitude of the potentials in *Nitella*? Since the changes in *Nitella* obey the equations of Nernst and of Henderson for diffusion potential and these equations enable us to predict the values found in various mixtures of electrolytes we may suppose that we are dealing with diffusion potentials. We can determine the relative values of u_K and v_{Cl} for the outer protoplasmic surface in *Nitella* (p. 550) and the P.D. across the protoplasm can be calculated from the activity gradient of KCl across the protoplasm (since the other electrolytes present have relatively little effect on the P.D.).

The sap in contact with the inner protoplasmic surface contains about 0.05 *M* KCl and if we put 0.001 *M* KCl outside the protoplasm we may write for 25°C., if $u_K = 73$ when v_{Cl} is taken as unity,

$$\text{P.D.} = 59 \frac{73 - 1}{73 + 1} \log \frac{0.05}{0.001},$$

where 0.05 and 0.001 are the concentrations (here used in place of activities) of KCl in the sap and in the external solution, respectively. This gives 97 mv. The average observed value is usually about 100 mv.

It would therefore appear that the diffusion potential of KCl in the sap is sufficient to account for the observed potential. On this basis we may expect to change the potential by changing the concentration of KCl in the sap. This can readily be done. If we place 0.24 *M* sucrose + 0.001 *M*

KCl at one end of the cell, called *A*, and 0.001 *M* KCl at another spot, designated as *B*, we see that water enters at *B*, moves through the cell and comes out at *A*. This produces a visible rush of particles in the vacuole from *B* to *A*. This movement of water carries KCl from *B* to *A* but KCl cannot pass through the protoplasm at *A* (except very slowly) and consequently it becomes more concentrated at *A* as water escapes into the external solution of sucrose. Hence the concentration of KCl increases at *A* and falls off at *B*. At the same time the potential increases at *A* and falls off at *B*. This can be shown by placing electrodes at *A* and *B* and connecting them to a third electrode, *C*, at another spot where the potential is kept constant and sap is prevented from moving.

When we replace the sucrose solution at *A* by 0.001 *M* KCl we see that water enters at *A*, moves through the cell and escapes at *B*, thus reversing the flow of sap. The concentration of KCl falls off at *A* and increases at *B* and in consequence the potential falls off at *A* and increases at *B*.

These experiments illustrate some of the methods used to determine the nature of bioelectric potentials. Although they have been developed especially in the study of large plant cells they can be applied to a considerable extent to other cells.

This study of resting potentials leads us to ask how these potentials are altered when an electrical disturbance travels along the cell. Since the preceding account has dealt with potentials in *Nitella* the simplest procedure is to continue with an account of electrical disturbances in this cell.

Stimulation.—This subject can not be discussed in detail but some of its fundamental features may be briefly mentioned. It consists of an electrical disturbance which is produced at one point and which usually travels along the cell.

Chemical Stimulation.—When the external solution is 0.001 *M* NaCl there is an outwardly directed potential of about 100 mv. at all points (Fig. 2). This is due to the diffusion potential of KCl at the non-aqueous film, *Y*, forming the inner protoplasmic surface. If we replace 0.001 *M* NaCl by 0.01 *M* KCl at *A* we create an inwardly directed potential of 100 mv. at the outer surface which balances the outwardly directed potential of 100 mv. at the inner surface and thus reduces the net potential at *A* to zero. At the same time the electrical resistance at *A* falls to about 1% of the normal, since both the inner and the outer surfaces of the protoplasm are in contact with a relatively high concentration of K^+ (the sap contains about 0.05 *M* KCl). All of these facts are established by direct measurement.

In this way a difference of potential between *A* and *B* is set up but if the cell is in a favorable state this difference soon decreases because the potential at *B* falls off in such fashion that *B* may be said to discharge into *A*. Then *C* discharges into *B* and in this way the disturbance travels

along the cell. During the discharge we may suppose that KCl moves out of the sap into the external solution, thereby lowering the outwardly directed activity gradient of K^+ on which the diffusion potential depends. There may be an actual rupture or a great increase in the permeability of the protoplasmic surface films at *B* when the electrical pressure reaches a certain point. If a film is 0.1 micron thick as may well be the case and the potential is 100 mv. the electrical gradient across the film just before the discharge is 10,000 volts per centimeter.

Electrical Stimulation.—It might be expected, in view of these facts, that when the external solution is 0.001 *M* NaCl and the potential 100 mv. at all points an e. m. f. applied by means of two electrodes in such fashion as to create a sufficient outgoing current at *B* and an ingoing current at *A* might

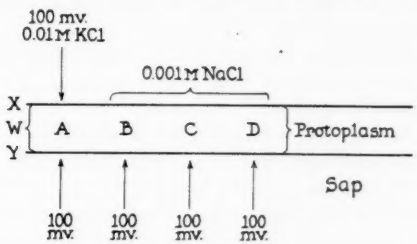

FIGURE 2

Diagram to illustrate the potentials assumed to be present in a *Nitella* cell. At the inner non-aqueous surface film, *Y*, there is a potential of 100 mv. at all points. At the outer film, *X*, no potential is assumed to exist when the external solution is 0.001 *M* NaCl but when this solution is replaced by 0.01 *M* KCl there is an inwardly directed potential of 100 mv. at *X* so that the net potential across the protoplasm is zero. No precise assumption is made regarding the concentration of KCl in the sap.

cause K^+ to move outward at *B* and reduce the potential at *B* so that the next spot, *C*, could discharge into it. This appears to be the case but it is necessary to apply more than 100 mv. since the resistance at *A* is now higher than in the former case. In describing chemical stimulation it was stated that the current entered *A* where it was in contact with 0.01 *M* KCl and the resistance was only 1% of that in a region in contact with 0.001 *M* NaCl, as in the present case.

Stimulation occurs with an outgoing electrical current which carries K^+ outward, not with an ingoing current which carries K^+ inward.

When K^+ moves out of the sap this movement is opposed by a process which is continually operating to drive K^+ inward and to keep its con-

centration inside the cell much greater than outside. The following suggestion may be made in regard to the process.

The experiments indicate that ions at the outer surface of the non-aqueous layer (surrounding the protoplasm) may combine with organic substances formed only at this spot and may then migrate through this layer to the aqueous protoplasm. If the organic substances are then irreversibly decomposed so as to set the ions free it is evident that as free ions they cannot pass out, except very slowly, since they are only slightly soluble in the non-aqueous surface layer. Hence they will be trapped and accumulate inside. We may assume that the reaction which decomposes the organic substances yields energy.

Recovery.—When K^+ has moved outward at *B* and the potential at *B* has fallen K^+ is moved back into the vacuole by the accumulative process. This is called recovery.[5]

Recovery involves a return to the sap of K^+ and other electrolytes which have moved outward. It also requires restoration to normal of surface films which have suffered change.

If an impulse arrives before recovery is complete there may be a loss of whatever potential has been recovered at that time but the magnitude of the change falls short of the normal response. For a short time after stimulation no response is possible ("absolute refractory period") but as recovery proceeds the response increases with progressive restoration of the outwardly directed activity gradient and outwardly directed diffusion potential at *Y*.

Propagation of Impulses.—When the potential at *A* falls to a certain value so that the next spot, *B*, discharges into *A* we see that *B* becomes an electrical sink into which the next spot, *C*, discharges and in this way the disturbance travels along the cell. The rate of travel in *Nitella* at 25°C. is usually about 2 cm. per second but may be much faster. In any case it is very much slower than in muscle and nerve.

If the traveling impulse reaches a spot (known as a "block"), which is unable to respond by a discharge, the impulse can progress no further. If we have three adjoining spots, *A*, *B* and *C*, with *A* and *C* normal and *B* serving as a block, the impulse can travel past *B* only if we connect *A* and *C* by a moist thread through which *C* can discharge into *A*. The impulse can then continue to travel along the cell. A gap between two cells can be bridged over in the same way by means of two moist threads which complete the circuit so that the impulse can travel from one cell to the other.

A block may consist of an area of very low potential or it may be a region which has the normal resting potential but does not discharge. Normal cells of *Nitella* can be rendered inexcitable by anesthetics or by leaching in distilled water. The leaching apparently removes from the cell a substance (called *R* for convenience) which makes the cell excitable. It also

ensures a high mobility for K^+ in the outer protoplasmic film and this produces the potassium effect. It can be extracted from the distilled water used for leaching and applied to the cell which then reacts normally. A substance which has the same restorative effect is found in blood and it seems possible that it plays an important part in maintaining the normal excitability of muscle and nerve.

Mechanical Stimulation.—Mechanical stimulation in *Nitella* presents a picture which differs from that hitherto described.

The most convenient way to produce mechanical stimulation is by pinching the cell. This sets up a compression wave which travels along the cell (presumably with the speed of sound in water) and causes a loss of potential at each spot it reaches. The effect may be reversible or irreversible depending on the violence of the disturbance.

If the cellulose wall is imbibed with 0.001 *M* NaCl there is an outwardly directed (positive) potential[6] everywhere and when the compression wave arrives at any point the loss of this positive potential causes a "negative variation." But if the cellulose wall is imbibed with 0.1 *M* KCl there is an inwardly directed (negative) potential at all points and when the compression wave arrives the loss of this negative potential causes a "positive variation."

It is evident that more investigation is needed at almost every point to clear up the mechanisms involved in stimulation. The energy relations are of interest since stimulation is accompanied by an increased output of CO_2 in nerve.

In conclusion it seems desirable to stress again the importance of the non-aqueous surface films. These structures, invisible under the highest magnification, play an all-important rôle. They are the seat of considerable electrical forces so that when a sufficient number of cells is in series an e. m. f. of 500 volts may be available, as in the electric eel. The high electrical capacity and resistivity of the cell is due to them. They regulate the intake and outgo of all substances and thus control metabolism. If they are destroyed death ensues at once. The behavior of such films involves the little understood laws of surface chemistry and physics and deserves intensive study.

The problems encountered in the study of *Nitella* are for the most part closely related to those we meet in experiments on muscle and nerve. Many other cells present different problems of equal interest and complexity. It is our hope that this conference may bring us more aid from physicists and physical chemists in dealing with these important questions.

[1] In some cases larger values are observed. In *Halicystis* we find 50 mv. The cause of this is not known.

[2] For convenience activities will be regarded as equal to concentrations in all cases in this paper.

[3] If the same standard state is assumed for the solution in both phases S would be unity, but it is more convenient to assume different standard states so that as long as the properties of the two phases remain constant S will be constant but as a rule its value will not be unity.

[4] The partition coefficient includes only the ions K^+ and Cl^-. It would not be constant if it included the undissociated KCl in X (but this undissociated part would not affect the diffusion potential), cf. Osterhout, W. J. V., *J. Gen. Physiol.*, **26**, 293 (1942–1943).

[5] If sufficient K^+ is present in the external solution there is no recovery since the accumulative forces do not produce a sufficiently great outwardly directed activity gradient of K^+.

[6] I.e., cations tend to flow across the protoplasm from the sap to the external solution.

SOME PHYSICAL ASPECTS OF BIOELECTRIC PHENOMENA

By Kenneth S. Cole*

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Communicated by D. A. MacInnes, July 8, 1949

The structure and activity of living systems present many problems of physics, chemistry and mathematics and the electrical aspects of these problems have been investigated almost continuously for the past century and a half. As increasingly powerful concepts, techniques and instruments have been developed they have been applied to the study of these phenomena and, as a result, we now have available a vast store of information on the bioelectric phenomena of organisms, tissues and cells—both plant and animal. Much of this information has been correlated into the general principles of electrophysiology and, in some cases, in surprisingly successful quantitative form. But the further description and interpretation of the various bioelectric phenomena in terms of physical and chemical concepts has been, in general, less successful. Although it has been almost impossible to design and execute experiments on living materials which disclose their physical characteristics in simple and direct form, some of these characteristics have been measured and found to be strikingly similar to phenomena of non-living systems. This is highly gratifying to the physical scientist and very useful to the biologist except for the several important instances in which the behavior of these non-living systems has itself not been satisfactorily explained. Thus, as is found for other biological phenomena, the physical aspects of the electrical phenomena may present problems of experiment and theory which are not confined to the living systems alone but extend into the physics and chemistry of non-living liquid and solid materials.

This contribution to the Conference on Bioelectric Potentials presents

some of the simpler electrical characteristics of living cell membranes which have come from the application of physical principles and techniques to the measurement and analysis of the electrical properties of cells and tissues. These membrane characteristics may serve the Conference both as background information and as examples of the many unsolved physical problems of biological systems.

Although the experimental methods used in these investigations of the electrical characteristics of the cell membranes cannot be discussed in detail they should not be ignored completely. The design of the experiments and the equipment and the analysis of the results have usually been rather straightforward applications of conventional techniques such as those of potential theory, circuit analysis, electrochemistry, and communication and electronic engineering. But as often as not the conditions imposed by living cells have required that the necessary techniques be pushed beyond those achieved in current practice and in some cases the equipment has had to reach to the natural limits of sensitivity and resolution.

Membrane of the Cell.—It is well established not only that the interior of the living cell is very different from the external inanimate environment in composition, structure and electric potential, but also that these differences are maintained by a barrier at the surface which is necessary for the life of the cell. Although this barrier may not be positively identified under the microscope—as for example a part of the plasma membrane—it is definitely recognized by other characteristics—such as a rather small permeability to water and to many solutes. The structure which constitutes the barrier to the free flows of ions in and out of the cell is most probably the seat of the immediate source of electrical energy and the origin of the principal bioelectric effects. This structure is our primary concern and for convenience we shall refer to it as the membrane of the cell.

Capacity of the Membrane.—The cell membrane was thought of electrically as a "leaky condenser" before 1900 and by 1925 the capacity of this condenser had been measured in the red blood cell[1] and found to be about one microfarad per square centimeter. Since that time the membrane capacity of an adequate number of cell types has been measured[2] with sufficient accuracy to justify the hypothesis that all living cells have a membrane capacity and that it is of the order of one microfarad per square centimeter. On the assumptions of a macroscopic behavior and a dielectric constant of three, the membrane has a thickness of 30 A. This thickness is clearly of molecular dimensions and it suggests the need for more detailed information and analysis on thin films. A further complication is that only a few of these membranes exhibit a pure static capacity, it being more usual to find a behavior which resembles the dielectric loss of engineering insulators.

The alternating-current characteristics of the membrane capacity are

conveniently considered[3] in two parts—C', the conservative or "real" component and C'' the dissipative or loss, component—which are combined as the complex capacity,

$$C^* = C' - iC'', \tag{1}$$

where $i = \sqrt{-1}$. The membrane capacities are adequately expressed empirically[4] by

$$C^* = \bar{C}(i\omega)^{-(1-\alpha)},$$

where $\bar{C}$ is constant, $\omega/2\pi$ is the frequency and α is a loss index, $0 \leq \alpha \leq 1$, which is related to the phase angle $\phi = \alpha\pi/2$. These results can be expressed in terms of a resistance and a capacity, both of which vary with frequency, or, as shown in figure 1, in terms of C' and C''.

This behavior of cell membranes is similar to the polarization of elec-

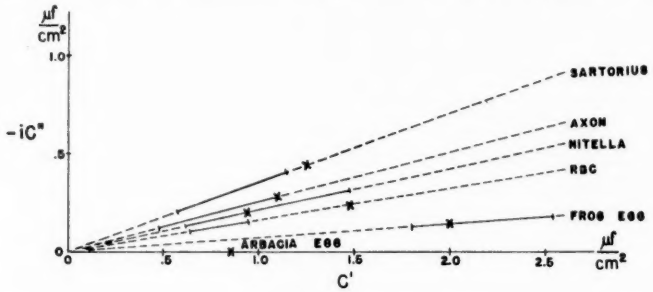

FIGURE 1

Complex capacity loci, C′ *vs.* C″, for various cell membranes; in each case the solid line represents the experimentally determined portion of the locus and X is the observed or calculated point for a frequency of 1 KC.

trode systems such as platinized platinum, silver–silver chloride and calomel, and this similarity has led to the attractive suggestion that this membrane capacity also is a phenomenon of ion transport. Although it may not be possible either to accept or reject the hypothesis without a better understanding of the electrode systems, several factors make it seem rather improbable:

(*a*) This analytical description of the membrane and electrodes is not unique to them but can be applied with equal success to the electrical properties of dielectrics[5] and a barrier layer photovoltaic cell[6] and to the mechanical characteristics of some viscoelastic materials.[7]

(*b*) The capacities of the electrode systems are usually one or more orders of magnitude larger than those of the cell membranes.

(*c*) Although the electrode characteristics depend to a considerable de-

gree upon the method of manufacture and the composition and concentration of the electrolyte, the membrane characteristics seem quite stable and remain relatively unchanged as the cell becomes active, is injured or is placed in a chemically modified medium.

(*d*) The anomalous reactance, to be mentioned later, has some characteristics which are more closely analogous to those found in ion transport processes than have these membrane capacities of one microfarad per square centimeter.

The membrane capacity may—as has been indicated—be dielectric in origin. For a dilute solution of dipoles the complex dielectric factor ϵ^* is given by

$$\epsilon^* = \epsilon - i\epsilon = \epsilon_\infty + (\epsilon_0 - \epsilon_\infty)/(1 + i\omega\tau), \qquad (2)$$

where ϵ_0 and ϵ_∞ are the dielectric constants at zero and infinite frequencies, respectively, and τ is the relaxation time.[8] An equivalent circuit is readily interpreted with one capacity for ϵ_∞, the deformation polarization, another capacity for $\epsilon_0 - \epsilon_\infty$, the rotation polarization, and a resistance for $\tau/(\epsilon_0 - \epsilon_\infty)$, the viscous dissipation in rotation. For concentrated solutions of dipoles and polar liquids and solids one often finds[5]

$$\epsilon^* = \epsilon_\infty + (\epsilon_0 - \epsilon_\infty)/[1 + (i\omega\tau)^{1-\alpha}]. \qquad (3)$$

In the equivalent circuit the resistance is now replaced by an impedance $z^* = z(i\omega)^{-\alpha}$. If now $\epsilon_\infty << \epsilon' << \epsilon_0$ and α is near unity the element z^* is the only one of importance and equation (3) approaches the membrane characteristic of equation (1) and figure 1. Then, since ϵ_∞ has a minimum value of unity, the real part of the dielectric factor ϵ', for the membrane must be considerably larger than unity. Furthermore, since the static dielectric constant, ϵ_0, in turn is much larger than ϵ' it must be at least ten and may be hundreds. In case the static dielectric constant is as low as ten, the membrane thickness then becomes about 100 A.

In the case of dilute solutions and those liquids and solids which follow equation (2) the dipole rotation is opposed by only a pure viscous resistance and $\alpha = 0$. But as the concentration of a solution such as beta lactoglobulin is increased,[9] or the temperature of a liquid such as glycerin[10] or a solid such as ice is decreased,[11] the value of α increases. This suggests a gradual replacement of the viscous opposition to rotation by intermolecular forces of an elastic nature which becomes almost complete for some solid polymers—and for the *Arbacia* egg membrane. The index α and the intermolecular forces may be expressed as a rather special distribution of relaxation times which varies from a single component for $\alpha = 0$ to an increasingly broad and flat distribution as α approaches unity.[5] If then the cell membrane capacity is of a dielectric nature and this interpretation of liquid and solid dielectrics is correct, the cell membranes are to be thought of as rather

solid structures in which the energy of thermal agitation is small or negligible in comparison with the intermolecular potential energy.

Conductance of the Membrane.—If equation (1) is a complete and correct description of its electrical characteristics, the cell membrane has no direct-current conductance and consequently is completely impermeable to ions. And indeed it has not been possible to demonstrate a conductance of the membrane with any certainty in the large number of experiments which have been done with small cells of irregular or poorly defined shapes and in media of rather high conductivity. As a result, serious consideration has been given to the possibility that materials can only be transported across the membrane as neutral molecules. However, by the use of long cells such as *Nitella*[12] and various nerve fibers,[13–15] a large cell such as frog egg in pond water[16] or an internal electrode as in *Valonia*[17] it has been possible to measure resistances ranging from 100 ohm-cm.^{2} to 100,000 ohm-cm.^{2} for the membranes of a number of cells in a resting state. Since membrane resistances as high as these could not have been detected in the other experiments it is possible to assume that all cell membranes have a similar conductance and ion permeability.[18]

When a cell becomes active,[19] is narcotized,[20] is subjected to changes in the composition of the external medium,[21] is injured or dying or is polarized by current flow[22] this membrane conductance changes. The conductance change correlates closely with the physiological state of the cell and may amount to several orders of magnitude while the change of the capacity, if any, is usually not more than 10 or 20%[19]. On this basis one may picture the capacity as characteristic of an ion-impermeable, inert structure composing usually more than 90% of the effective membrane area with the functional ion permeable aspect relegated to the small remaining area of the membrane.

On the assumption that the membrane has a thickness of 100 A its specific resistance is about 10^{10} ohms-cm or about 10^8 times that of protoplasm and the normal environments of many cells. Although this high resistance may be a result of either a very low concentration or a very low mobility of the ions in the membrane, it is more probably that both of these factors are involved to a considerable extent.

It is to be expected that the resting potential and the conductance of the membrane are both functions of the ion permeability and so should be closely related. At the present time there are only a few cells in which the resting potential has been measured in the absence of current flow and practically none in which the resting potential and the membrane conductance have been measured simultaneously.

The indications in the squid axon[21] are that the membrane resistance decreases for all increases of external potassium concentration, although the membrane potential is but slightly altered until the external potassium is

about three times its normal value. Changes of the external calcium seem to be almost without effect on the potential but the resistance becomes very high for a tenfold increase and practically vanishes as the external calcium is reduced toward zero. A more extensive use of internal electrode techniques should produce more satisfactory and complete evidence as to the nature of the relation between these two characteristics of the cell membrane.

Rectification of the Membrane.—The current flow through a membrane is proportional to the change of the potential difference for small changes— a few millivolts for the squid axon—and the ratio of current to potential change is quite properly expressed as the conductance discussed above. But several lines of evidence have made it highly probable that the relationship is not so simple for larger changes of potentials as has been verified by direct measurements on nerve fibers[15, 22] and plant cells.[23] As the potential difference was decreased more than a few millivolts the outward current increased more rapidly than it did for small changes, while for the large increases of the potential difference the inward current increased more slowly than for the small changes of potential. This highly non-linear relationship between the current and the potential difference is of the type which characterizes a rectifier. In the squid axon the ratio of the maximum conductance for outward flow to the minimum conductance for inward flow is about a hundred to one.[22] If this effect is ascribed to an ion, such as potassium, which has a higher concentration inside than out, the concentration of this ion should be higher for an outward than for an inward current flow at every point in the membrane. As a result the outward conductance should be higher than the inward which is in qualitative agreement with the experiments.

The problem may be approached quantitatively by means of the equation for the ion concentration, n, under the influence of diffusion and electric forces,

$$\rho \frac{\partial n}{\partial t} = - \frac{\partial}{\partial x}\left(neX - kT \frac{\partial n}{\partial x}\right), \tag{4}$$

where ρ is the friction coefficient and X is the electric field. This equation has been applied to the barrier layer rectifier and solved on the assumption of a negligible space charge. Although this assumption does not appear to be justified in the present problem and a more rigorous solution is not available, it has been possible to obtain reasonably good agreement with some of the experimental results on the rectification characteristics and the membrane potential of the squid axon.[24]

Anomalous Reactance of the Membrane.—When definitive data were taken on the membrane of squid axon at frequencies below 1 kc. another physical characteristic made its appearance.[25] This is a reactance in addition to the

membrane capacity of one microfarad per square centimeter which, unlike the latter, is closely correlated with physiological and chemical factors and may be either capacitative or inductive.[19] The present indications are that it is normally present in the squid axon membrane and somewhat capacitative although it is rather variable. A cursory inspection of the data on other biological systems suggests rather strongly that this anomalous reactance is not a unique feature of the squid axon membrane although indications of an inductive reactance have not been found except in nerve fibers.

The reactance of the squid axon membrane becomes highly capacitative with excess calcium in the external medium and highly inductive as the normal calcium is removed from the medium. An excess of external potassium up to five times the normal value gives rise to an inductive reactance which then disappears for still higher concentrations. The investigations of the effect of membrane current flow on this anomalous reactance have not been satisfactory but the preliminary results indicate that the reactance tends to become inductive for outward current flow and capacitative for an inward flow.

The most attractive explanation of this reactance[3] is that it is a perfectly general consequence of the non-linear characteristic of the membrane. Let us assume that the mechanism which is responsible for the non-linearity of a system cannot respond to instantaneous or sufficiently fast variations. In electrical terms we have $e = r_\infty i$, where e and i are small rapid variations of the potential difference and the current, respectively, and r_∞ is the instantaneous variational resistance. We will now assume that this instantaneous response is modified by the non-linear mechanism to approach the steady-state response, $e = r_0 i$ at a rate $-\beta(e - r_0 i)$, where r_0 is the steady state of variational resistance and β is the rate constant of the mechanism. The phenomenon is then described by the equation

$$\frac{de}{dt} = r_\infty \frac{di}{dt} - \beta(e - r_0 i).$$

The transient solution for a constant current i_0 applied at $t = 0$ is

$$e(t) = [r_0 - (r_0 - r_\infty)e^{-\beta t}]i_0$$

and the steady-state impedance is

$$z(i\omega) = r_\infty + \frac{r_0 - r_0}{1 + i\omega\tau},$$

where $\tau = 1/\beta$. According as $r_0 - r_\infty$ is greater or less than zero both the transient and the steady-state solutions are represented by equivalent circuits containing resistances and a single capacity or inductance, respectively. The values of the reactive elements for one circuit are

$$C = \tau/(r_0 - r_\infty) \text{ and } L = (r_\infty - r_0)\tau.$$

This formal consideration indicates that an anomalous reactance is to be expected in a non-linear system, but for an interpretation of the velocity constant it is necessary to make a detailed analysis of the mechanism involved. Although either a chemical reaction or a transport process may be primarily responsible for this velocity constant we shall use only the simple potassium diffusion model already discussed as an illustration.

In this model, the distribution of ions at equilibrium is such that the outward diffusion is prevented by the inwardly directed electrical field. At the instant when the field is changed by a sudden increase or decrease of the potential difference across the membrane the initial current flow is determined by the ion distribution which was present before the potential was changed. But as time goes on the ion distribution and the current change to approach the steady-state values required by the alteration of the potential. For an increase of potential, the current decreases from its initial value to a lower steady-state value which corresponds to the presence of an equivalent capacity in the membrane. For a decrease of potential, the current rises to a higher value indicating an equivalent inductance. The details of these processes have been calculated from equation (4) and it is found, as can be seen by normalizing the equation, that the order of magnitude of velocity constant is given by

$$\beta = X^2e^2/\rho kT.$$

The available data indicate that β is of the order 100 sec.$^{-1}$ for the squid axon. A crude combination of this value and the conductance data leads to an ionic concentration in the membrane $n = 5.10^{17}$ ions/cc. or about 0.001 N and a friction coefficient $\rho = 3.10^{-4}$ dyne sec./cm. which is larger than the value for the potassium ion in water by a factor of about 10^5.

Conclusion.—Although this discussion has been limited to a few of the simpler physical aspects of bioelectric phenomena, it is apparent that no one of the membrane properties mentioned has as yet been adequately explained in terms of physical or chemical principles and that the description of the structure and function of the living cell membrane in elementary terms is still far from complete.

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[1] Fricke, H., *Phys. Rev.*, **26,** 682–687 (1925).

[2] Cole, K. S., *Tabulae Biologicae* (*Cellula*), **19** (2), 24–47 (1941).

[3] Cole, K. S., Four Lectures on Biophysics, Rio (1947).

[4] Cole, K. S., *J. Gen. Physiol.*, **15,** 641–649 (1932).

[5] Cole, K. S., and Cole, R. H., *J. Chem. Phys.*, **9,** 341–351 (1941). Cole, K. S., and Cole, R. H., *Ibid.*, **10,** 98–105 (1942).

[6] Wood, L. A., *Rev. Sci. Inst.*, **4,** 434–439 (1933).

[7] Nolle, A. W., Quarterly Report Acoustics Lab., MIT, April (1947).

[8] Debye, P., Polar Molecules, Chemical Catalog Co., N. Y. (1929).
[9] Shaw, T. M., Jansen, E. F., and Lineweaver, H., *J. Chem. Phys.*, **12**, 439–448 (1944).
[10] Morgan, S. O., *Trans. Electrochem. Soc.*, **65**, 109–116 (1934). Wintsch, H., *Helv. Phys. Acta.*, **5**, 126–144 (1932).
[11] Murphy, E. J., *Trans. Electrochem. Soc.*, **65**, 133–142 (1934).
[12] Blinks, L. R., *J. Gen. Physiol.*, **13**, 495–508 (1930).
[13] Cole, K. S., and Hodgkin, A. L., *Ibid.*, **22**, 671–678 (1939).
[14] Hodgkin, A. L., and Rushton, W. A. H., *Proc. Roy. Soc.*, **B133**, 444–479 (1946).
[15] Hodgkin, A. L., *J. Physiol.*, **106**, 305–318 (1947).
[16] Cole, K. S., and Guttman, R. M., *J. Gen. Physiol.*, **25**, 765–775 (1942).
[17] Blinks, L. R., *Ibid.*, **14**, 139–162 (1930).
[18] Cole, K. S., *Trans. Faraday Soc.*, **33**, 966–972 (1937).
[19] Cole, K. S., and Curtis, H. J., *J. Gen. Physiol.*, **22**, 37–64 (1941). Cole, K. S., and Curtis, H. J., *Ibid.*, **22**, 649–670 (1939).
[20] Guttman, R., *Ibid.*, **22**, 567–591 (1939).
[21] Cole, K. S., and Marmont, G. H., *Fed. Proc.*, **1** (II), 15–16 (1942).
[22] Cole, K. S., and Curtis, H. J., *J. Gen. Physiol.*, **24**, 551–563 (1941). Cole, K. S., and Baker, R. F., *Ibid.*, **24**, 535–549 (1941).
[23] Blinks, L. R., *Ibid.*, **13**, 793–806 (1930).
[24] Goldman, D. E., *Ibid.*, 37–60 (1943).
[25] Cole, K. S., and Baker, R. F., *Ibid.*, **24**, 771–778 (1941).

THE SOURCE OF THE BIOELECTRIC POTENTIALS IN LARGE PLANT CELLS

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The use of large plant cells for the study of bioelectric potentials was originated by Professor Osterhout, and received much of its advance in his laboratory.[1] The advantages of such multinucleate cells are several: they occur either singly, or easily separable from their neighbors with a minimum of dissection or injury, and they survive well in the laboratory, often for days or even weeks with a fine glass tube making connection with the cell sap. The large and measurable surface allows expression of resistance and capacity in definite terms; the capacity being often as high as several microfarads in a single cell, and resistances as high as one megohm (100,000 ohms per square centimeter of surface). New solutions may be quickly applied over the whole surface, or at definitely separated areas for the study of concentration effects, etc. The cell sap may be analyzed for its constituents, or obtained in sufficient quantity to apply to the exterior (see below); in two genera it may be replaced within the vacuole by perfusion with sea water or other new solutions. Through these means the

effect of ionic and other changes has been studied at both the outer and inner (or vacuolar) protoplasmic surfaces, leading to a better understanding of the gradients, and the origin of the bioelectric potential.

Normal P.D.—Some nine or ten genera of large cells have now been studied. The results are tabulated in table 1. The majority display a normally positive potential difference (P.D.) across the protoplasm, the outer surface being positive to the external measuring circuit. As in the case of many animal cells *Halicystis, Bryopsis, Batophora, Nitella, Chara* and *Hydrodictyon* all display positive potentials of 50 to 80 mv. (*Nitella* sometimes higher). However, *Valonia*, perhaps the most studied genus, dis-

TABLE 1

POTENTIAL DIFFERENCE (P.D.) ACROSS THE PROTOPLASM OF MULTINUCLEATE PLANT CELLS

The sign is that of the outer solution; when positive, the current tends to flow outward across the protoplasm to the outer solution and to the measuring instrument. Values in millivolts. ( ) signifies few observed values; (?) questionable.

ORGANISM	P.D. IN NORMAL ENVIRONMENT (SEA WATER OR POND WATER), MV.		P.D. IN NATURAL OR ARTIFICIAL SAP, MV.	
Marine:				
Valonia macrophysa	5 to 10	−	20 to 80	−
V. ventricosa	10 to 20	−	20 to 80	−
Ernodesmis verticillata	5 to 10 (?)	−		
Chamaedoris annulate	(5)	−		
Batophora Oerstedi	60 to 80	+	(5 to 10)	+
Halicystis ovalis	75 to 80	+	20 to 60	+
H. Osterhoutii	65 to 70	+	40 to 60	+
Bryopsis hypnoides	(50)	+	(0)	
B. corticulans	50 to 60	+	(0)	
Fresh water:				
Nitella flexilis	100 to 200	+	0 to 20	−
N. mucronata	100			
N. elevata	100 to 150	+		
Chara coronata	75 to 100	+		
Hydrodictyon patenaeforme	50 to 60	+	0 to 10	−

plays a low negative P.D. of 8 to 15 mv. in the sea water that is its natural environment. Its close relatives *Ernodesmis* and *Chamaedoris* are likewise negative. However, both these negative and positive P.D.'s are reproducible, not only as to sign, but even as to magnitude.

Application of Sap: Protoplasmic Asymmetry.—Since the vacuolar sap of most of these plants differs from the external solution (especially in its potassium content), it is often supposed that the resulting ionic gradients account for the P.D. If so, the latter should become zero when natural or artificial sap is applied externally. Table 1 shows that this is seldom the case. It is most nearly true in *Nitella* and *Bryopsis*, although even in these

genera the P.D. is sometimes reversed to slightly negative values by this treatment. In most other genera the P.D. is only slightly lowered, as in *Halicystis*, or is even *increased*, as in *Valonia*. Obviously, in the apparently symmetrical system:

Sap | Protoplasm | Sap

there must be some residual asymmetry within the protoplasm itself, as originally emphasized by Osterhout, *et al.*[2] The simplest assumption is that the two protoplasmic surfaces differ in their response to identical solutions. The nature of this response will be next considered.

External Cation Effects.—The outer surface of the cell has long been known to respond electrically to changes in the ion concentration or composition of the medium. Although various causes have been ascribed to these responses (e.g., phase boundary potentials, adsorption effects, colloidal alterations or effect upon metabolism), the most generally useful interpretation has been based on diffusion potentials through the protoplasmic surface or "plasma membrane," likewise taking into account the differing partition coefficients (S) of salts in this surface—presumably a non-aqueous phase, since it is not miscible with the cell sap or with water. These effects are clearly very different from those in water, since KCl displays practically no diffusion potential in water, but has a marked influence on the bioelectric potential of most plant cells, increasing in a regular fashion with concentration. In *Nitella* this is perhaps the most striking,[3] where the P.D. change plotted against log of concentration of KCl gives a straight line over a wide range of concentration (0.001 M to nearly 0.1 M). The slope of this line is nearly 58 mv. per tenfold dilution, or close to the limit for a perfect "potassium electrode." If the Nernst equation for diffusion potentials is applied, a relative mobility of K^+ ion equal to 73 must often be assumed to account for this slope, if that of Cl^- is taken as 1.0. The relative mobility of Na^+ in *Nitella* is ordinarily much smaller, usually between 2.0 and 3.0 when Cl^- is unity; this gives a concentration effect of 20 to 30 mv. per tenfold dilution. In both these cases dilute solutions are more positive to the measuring circuit than are more concentrated ones; consequently the normally positive P.D. of *Nitella* is decreased by higher concentrations of either KCl or NaCl. Other cations such as Li^+ and Cs^+ resemble Na^+; Rb^+ is nearly as high as K^+; while NH^+_4, Ca^+, and Mg^+ lie between these (see table 2). On going from one salt to another, it is frequently necessary to assume a different solubility or partition coefficient (S) to account for the change of potential. With this addition mixtures of two salts may also be calculated with the Henderson equation.[4]

In contrast to *Nitella*, in both of the marine genera most studied (*Valonia* and *Halicystis*), the relative mobility of Na^+ must be assumed as *less* than that of Cl^-, since dilution of the sea water (largely NaCl) causes the

P.D. to become less positive (more negative in the case of *Valonia*, with a negative P.D. already). This dilution effect is especially reproducible in *Valonia*,[5] where a regular stepwise increase of P.D. occurs on successive two-fold dilutions of sea water (or of the NaCl of sea water) with isotonic glycerol, up to $^1/_8$ the normal concentration. A straight line results when the P.D. change is plotted against log (1/ concentration), the slope being such as to demand a relative mobility of 0.114 to 0.33 for Na^+ when Cl^- is unity. Somewhat similar values result in *Halicystis* for Na^+, though the cells tolerate less dilution, and the P.D. changes are transient, with the time course exhibiting an extremum or cusp.

TABLE 2

RELATIVE MOBILITIES OF IONS IN OUTER PROTOPLASMIC SURFACE OF SEVERAL PLANT CELLS

(Calculated with the Nernst and Henderson equations on the assumption that the salt effects are due to differing relative mobilities in the surface.) Mobility of chloride ion assumed as unity. The figures enclosed in parentheses represent values obtained under special conditions.

ION	*Valonia macrophysa*	*Halicystis (Osterhoutii* OR *ovalis)*	*Nitella flexilis*
Cations:			
K	18 to 20	16 to 18	73 (8.7) (2.2)
Na	0.2 to 0.11	0.2 to 0.3	2.2 (8.7)
Li	0.2	0.2 to 0.3	2.04
Cs	0.2	0.2 to 0.3	1.72
Rb	18	16 (0.3)	73 (?) (8.76)
NH_4	(37)	(16?)	4.05
Ca			7.5
Mg		1.9	20.7
Anions			
Cl	1.0	1.0	1.0 (assumed as unity)
Br		(0.5)	
NO_3	(0.2?)	0.1	
Formate, acetate, etc.	(0.2?)	0.1	

Conversely, in both marine genera, K^+ appears to have a higher mobility than Cl^-; an *increase* of the originally low KCl content of sea water (doubling, quadrupling, etc.) tending to make the P.D. become less positive (or more negative in *Valonia*). But instead of the approximate independence of the time in the regular stepwise effects just mentioned, the time course is strongly cusped, with a prompt decline and a slow recovery. Damon[6] has interpreted this time course as due to the initial diffusion potential of KCl into the protoplasmic surface, followed by the rather rapid entrance of KCl into and across that surface, with consequent abolition of the diffusion gra-

dient, and loss of the diffusion potential. The same transient time course has been found with KCl increase on *Halicystis* by the author,[7] who now prefers to interpret the cusp as due to a "desensitization" of the surface, which ceases to discriminate between Na^+ and K^+ after 5 to 10 minutes' exposure to the latter. (Temperature influences this,[7] as does pH.[6]) In either case, by taking the maximum value of the cusp, it is possible to calculate an approximate value for the relative mobility of K^+, which is between 16 and 20, when that of Cl^- is again assumed as unity.

Although it may well be true that the high mobility of K^+, and its high concentration in the cell sap, accounts for the natural P.D. of *Nitella*, this can hardly be the explanation of the P.D. in *Valonia* and *Halicystis*. In these genera: (*a*) the potassium effects are transient, giving rise to no very permanent potential change; (*b*) the P.D. of *Valonia* is in the wrong direction for the gradient to be operative; (*c*) in *Halicystis* there remains a large positive P.D. when sap is applied externally,[7] when sea water is perfused in the vacuole,[8] or in a species (*H. Osterhoutii*) naturally containing less K^+ than the sea water itself.[9]

We must conclude that some other gradient must be responsible, and turn to experiments with other ionic species. The most fruitful investigations have concerned anion substitutions.

Anion Substitutions in Sea Water.—The most abundant anion of sea water, next to Cl^-, is SO_4^{--}, which reaches a concentration of about 0.028 *M* whereas Cl^- averages about 0.550 *M*. In both *Valonia* and *Halicystis* sulfate is absent from the cell sap, but its gradient to sea water is not very significant; it may be doubled, or omitted from an artificial sea water, with little effect upon the P.D., showing that it is not important *as such* in the normal environment. However, when the sulfate concentration is considerably increased, the P.D. of *Halicystis* is depressed, and a complete substitution of SO_4 for Cl in an artificial "sulfate sea water" produces a very striking effect:[10] the normally positive P.D. of some 80 mv. is reversed to a negative one of 20 to 40 mv.—a total change of over 100 mv. This is not transient, but is well maintained at this negative level for an hour or more. If such a pure sulfate sea water is now diluted with isotonic glycerol, the P.D. becomes less negative, indicating that the SO_4^{--} ion has a lower mobility than has Na^+ (which in turn is lower than that of Cl^-). If the mobility of Cl^- is again assumed to be unity, that of Na^+ is 0.3 and that of SO_4^{--} between 0.1 and 0.2.

Mixtures of sulfate and chloride sea waters give intermediate values, which are very stable, and roughly predictable from the Henderson equation for mixed electrolytes. As might be also predicted, it is possible to make an "indifferent" mixture of these two anions which gives practically no change on dilution with isotonic glycerol.

The behavior of sulfate is paralleled by most of the other anions tolerated

by the cells (except bromide, which resembles chloride, though it is somewhat less mobile). Thus nitrate, formate, acetate, propionate, lactate, pyruvate, fumarate, succinate and malate all behaved essentially like sulfate; so did a "sea water" made of the amino-acid salt, sodium glutamate. (Oxalate and citrate were toxic due to their effect on the necessary calcium.) Nitrate and formate were most studied, being most constant and least toxic. Both these anions, when substituted for chloride, maintained the P.D. at a very constant negative value of about 40 mv. Mixtures with normal sea water, containing 0.1 *M* NO_3, 0.2 *M*, 0.3 *M*, etc., maintained predictable intermediate levels. A mixture of 4 or 5 parts NO_3 to one of Cl gave an indifferent solution showing little or no change on dilution with glycerol, the negative influence of the NaCl dilution being balanced by the positive one of $NaNO_3$ dilution. Dilution of pure nitrate sea water showed the relative mobility of NO_3^- ion to be about 0.1 when that of Na^+ is 0.3 and that of Cl^- is unity. Since the mobility of K^+ is 16 or more, we have the remarkable alternating sequence: $K > Cl > Na > NO_3$, etc. Clearly no membrane with charged pores could give rise to this alternation of mobilities for positive and negative ions. That nitrate has a low actual as well as relative mobility in the cell surface is indicated by the increased resistance of cells in contact with it, and by the very persistent P.D. changes produced by it (instead of the cusps produced by the very mobile K^+ ion).

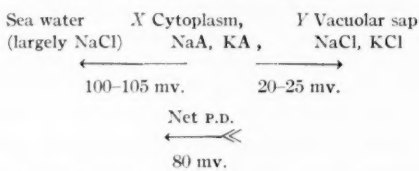
Source of Potential.—Since a great variety of organic anions behave like nitrate and sulfate in having a very low relative mobility in the outer protoplasmic surface, we may now postulate a possible source of the normal potential. All we require is to have the total organic anions of the cytoplasm approximate 0.5 *M:* in this we may include not only the fatty acids, lactate, pyruvate, etc., actually studied, but a variety of amino acids, and of course proteins, whose anions would doubtless have an even lower mobility in the protoplasmic surface. Analytic data on the very thin layer of cytoplasm in these cells are lacking, but it is hoped to obtain them from the related genus *Codium;* some estimates in other cells make the assumption at least a possible one. But even if such a gradient should set up a large potential across the outer surface, we must know the electrical situation across the vacuolar membrane as well; if the latter behaves like the outer surface, an oppositely directed potential would be set up cancelling the one at the outer. Fortunately, it is possible to learn something about the vacuolar surface in *Halicystis* and *Bryopsis*.

Vacuolar Perfusion.—This is accomplished by direct substitution of new solutions in the vacuole, either by two inserted capillary tubes[8] (in *Halicystis*) or by cutting both ends of the cell (in *Bryopsis*). In the latter case the substitution can be accomplished in about a minute; in *Halicystis* this requires 5 to 10 minutes (as shown by tracer dyes). In both cases the replacement of natural sap, fairly rich in KCl, by sea water, has practically no effect upon

the P.D. That of *H. ovalis* remains still at 80 mv. positive, despite the absence of any net gradient across the protoplasm, in the apparently symmetrical system:

Sea water | Protoplasm | Sea water.

Clearly again (just as with the external application of sap) a KCl gradient between sap and sea water is not responsible for the potential difference, which must be referred to an internal asymmetry of the protoplasm. The nature of this becomes apparent when anions are changed in the perfusing fluid. When nitrate is substituted for chloride, either in artificial sap or sea water, there is indeed an effect on the P.D., which *increases* from its normal 80 to 100 mv. or more positive. This is to be expected from the low mobility of the NO_3^- ion, compared to Cl^-. But the amount of change is only 20 to 30 mv., instead of the 100- to 120-mv. change produced by this same substitution externally. Evidently the inner or vacuolar surface is able to discriminate only $^1/_4$ to $^1/_5$ as well as the outer surface between Cl^- and NO_3^-. Formate and several other organic anions again behave like nitrate when perfused. We may therefore conclude that in the scheme suggested below the potential established across the surface (Y) by the organic anions of the cytoplasm will be only $^1/_4$ to $^1/_5$ that set up across the outer surface (X). Assuming the concentration of these anions (A) again at about 0.5 M, we might expect the following values:



The net P.D. is obviously due to the same gradient operating across two different membranes, one of which develops a diffusion potential four or five times as large as the other (and of course in opposite directions).

Reversal of P.D.—Independent evidence for the existence and magnitude of two such discrete potentials, giving an algebraically additive total P.D., comes from the effect of agents apparently altering the outer surface independently of the inner. Dilute ammonium salts are most reproducible in this respect: at a threshold of between 0.001 and 0.003 M NH_4Cl in sea water, the P.D. of *Halicystis* suddenly decreases and then reverses in a very characteristic time course.[11] Raising the pH from 8.0 to 9.0 has the same effect in the presence of lower NH_4Cl concentrations. This indicates that the penetration of undissociated ammonia is responsible for the effect, the "all-or-none" nature of which suggests the complete loss of the positive potential at the outer surface (X). That at the inner surface (Y) inwardly

directed, persists at 20 to 30 mv. negative, and is not influenced by further increases of ammonia. It is possible that the entering ammonia, having passed across the lipoid outer surface, combines with the polar groups of the latter, which are oriented toward the cytoplasm. This must saponify, emulsify or otherwise alter the outer surface, so that it loses its power to discriminate between various ions. (Since the resistance rises, it is possible that all ionic mobilities are decreased, to become nearly equal.) This is borne out also by the fact that under ammonia treatment, the outer surface is no longer responsive to anion substitutions: nitrate sea water has no effect when the P.D. is reversed by the weak base. (Various amines, pyridine, aniline and several alkaloids behave like ammonia in producing this effect.) The inner or vacuolar surface, on the other hand, seems to be immune, since the inwardly directed potential persists even under much higher concentrations of NH_4Cl, or when the latter is perfused in the vacuole. The effect on the outer surface is quite reversible, normal positive P.D. returning when ammonia is withdrawn (or the pH lowered).

Another agent which causes reversal of P.D. is physiological unbalance of salts bathing the outer surface: if NaCl is substituted for sea water, the P.D. of *Halicystis* reverses temporarily to 20–30 mv. negative, before coming to zero.[12] Addition of calcium causes recovery. Apparently the outer surface (X) is first altered by lack of calcium, with loss of its potential, and uncovering of the negative potential at the surface Y before the latter is in turn affected.

Metabolic Effects: Lack of Oxygen.—It is well known that many tissues respond to critically low partial pressures of oxygen by a decrease of their bioelectric potential; *Halicystis* is no exception. When the surrounding sea water is bubbled with 1% or less O_2 in N_2, the P.D. falls from about 80 mv., to about 10 mv. positive.[13] The anion substitution technique again supplies the reason for this. When nitrate is substituted for chloride (O_2 being kept low) there is practically no effect on the P.D.; under anaerobic conditions the surface has ceased to discriminate between these two anions. Consequently it must have ceased to give a potential due to their gradient between cytoplasm and sea water. This accounts sufficiently for the fall of potential, even as to time course, since the nitrate effect disappears *pari passu* with the fall of potential under anoxia, and recovers along with the P.D. when oxygen is readmitted. The resistance rises markedly as the P.D. falls. The reasons for this effect of anoxia are interesting, implying some close connection between the cytoplasm and its surface, between the products of metabolism and the electrical properties of the cell membrane. (It is probably not necessary to assume a rapid change of the ionic gradients themselves, but only of the phase which gives electrical expression to them.) This might consist of the loss of some substance constantly produced in aerobic metabolism (see below), or the increase of some anaerobic fermenta-

tion product. It is possibly due to an increase of cytoplasmic acidity (e.g., lactic acid), for much the same effects are produced by weak acids which penetrate the protoplasm, such as carbonic or acetic: the P.D. likewise falls, the anion effect disappears and the resistance rises.

Other Chemical Effects.—The membrane properties can be affected by a variety of other substances, some in considerable dilution. Thus a number of weak acids and several phenolic substances (phenol, cresol, guaiacol) strongly influence the P.D. of *Valonia*, rendering it highly positive (like the normal P.D of *Halicystis*). Osterhout[14] found that this positivity was accompanied by a marked change of ionic mobilities in the protoplasmic surface, Na and K reversing their usual positons: thus if the mobility of Cl^- was assumed to be 1.0, that of K^+ became 0.36 (instead of 20), and that of Na^+ 4.5 instead of 0.3 (see table 2). In *Halicystis* exposed to such agents, Na was also raised above Cl, although K was not affected.[15] The direct-current resistance of both *Valonia* and *Halicystis* rises under these treatments, and marked polarization curves appear (likewise under anoxia) indicating that a change of absolute, as well as of relative, ionic mobilities has occurred in the protoplasmic surfaces. Benzene, nitrobenzene, dinotrophenol, hydroquinone, etc., have somewhat similar effects, possibly through alteration of the pathways of normal respiration, and the accumulation of certain intermediate metabolites. Cyanide and urethane lower the P.D. of *Halicystis* somewhat, but not as drastically as several of the treatments mentioned. Indeed, the actual *rate* of aerobic metabolism does not seem to be directly correlated with the P.D. Thus low temperatures, though decreasing respiration (with a respiration quotient for a 10° interval, Q_{10}, of 2 or more), scarcely lower the P.D. which has in *Halicystis* a Q_{10} of 1.2, i.e., a 20% decrease for a 10° interval, or about that of diffusion. In fact, the P.D. of *Valonia* may even become higher at low temperatures, probably because the entrance of K is slowed down, with abolition of the cusps of the KCl curves (see above) allowing the KCl of sea water to be more effective.[7] Nor does increased temperature affect P.D. as much as it does respiration. A direct dependence of P.D. on respiration is not indicated.

Effects of Oxidants and Reductants.—Some theories[16] have emphasized a direct connection between respiration and bioelectric potentials, via oxidation-reduction potentials as such. This implies that a continuous phase of the cell itself, not appreciably short circuited, is interposed between two regions of different redox potential, and can act as an electron acceptor and donor, like a gold or platinum electrode, to make such potentials manifest in a measuring circuit. The only such continuous phases which are known are the protoplasmic surfaces. Those accessible to experimental treatment, in *Valonia* and *Nitella* do not so function.[17] To test this experimentally, solutions bathing *Nitella* were poised with a variety of oxidants or reductants ranging from ferricyanide ($E_h = +0.454$ volt) to reduced safranin

(−0.288 volt) a total range of 0.740 volt in redox potential. Intermediate systems included ascorbate and dehydro-ascorbate; di-chloro-phenol-indophenol; and indigo-disulfonate. When such systems were oxidized or reduced, with individual redox changes of 200 to 300 mv. none of them showed any appreciable effect upon the P.D. of *Nitella*. (The cells responded normally, however, to different concentrations of NaCl or KCl.) *Valonia* has proved likewise indifferent, except when very powerful oxidants such as permanganate or peroxide were employed; these do slowly alter the P.D., probably through direct attack on the membrane substance, which then shows altered ionic responses. In view of the large response given by various concentrations of neutral salts, both inorganic and organic, it seems unnecessary to invoke redox potentials themselves as the cause of bioelectric potentials. This is in agreement with the chemical difficulties met in considering lipoid or other membrane constituents as electron donors or acceptors.

Summary.—Bioelectric potentials may be considered as due to gradients of inorganic and organic ions between protoplasm and sap or environment, coupled with protoplasmic surfaces across which the ions have markedly different mobilities, giving rise to diffusion potentials. The outer and vacuolar surfaces have different properties, giving rise to electrical asymmetries. The high mobility of K^+ may be responsible for the P.D. in some cases (e.g., *Nitella*), but the very low mobility of organic anions seems important in others (e.g., *Halicystis*). The properties of the surface are under control, anoxia, ammonia and phenolic substances being especially effective in altering the apparent mobilities of ions through them. Oxidation-reduction potentials do not seem to be directly involved.

[1] For reviews of earlier work from his laboratory, see Osterhout, W. J. V., *Bull. Natl. Research Council*, No. 69, 170–228 (1929); *Biol. Rev.*, **6**, 369–411 (1931); *Ergeb. Physiol. exptl. Path.*, **35**, 967–1021 (1933). References to earlier work are given therein.

[2] Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.*, **11**, 193–205 (1927).

[3] Hill, S. E., and Osterhout, W. J. V., *Ibid.*, **21**, 541–556 (1938).

[4] Hill, S. E., and Osterhout, W. J. V., PROC. NATL. ACAD. SCI., **24**, 312–315 (1938).

[5] Damon, E. B., and Osterhout, W. J. V., *J. Gen. Physiol.*, **13**, 445–457 (1930).

[6] Damon, E. B., *Ibid.*, **16**, 375–395 (1932); **21**, 383–409 (1938).

[7] Blinks, L. R., *Ibid.*, **16**, 147–156 (1932); **25**, 905–916 (1942).

[8] Blinks, L. R., *Ibid.*, **18**, 409–420 (1935).

[9] Blinks, L. R., and Jacques, A. G., *Ibid.*, **13**, 733–737 (1930).

[10] Blinks, L. R., *Cold Spring Harbor Symposia Quant. Biol.*, **8**, 204–215 (1940).

[11] Blinks, L. R., *J. Gen. Physiol.*, **17**, 109–128 (1933).

[12] Blinks, L. R., Rhodes, R. D., and McCallum, G. A., PROC. NATL. ACAD. SCI., **21**, 123–125 (1935).

[13] Blinks, L. R., Darsie, M. L., and Skow, R. K., *J. Gen. Physiol.*, **22**, 255–279 (1938).

[14] Osterhout, W. J. V., *Ibid.*, **20**, 13–43 (1936).

[15] Osterhout, W. J. V., *Ibid.*, **21**, 707–720 (1938).

[16] Lund, E. J., *J. Exp. Zool.*, **51**, 265–337 (1928).

[17] Blinks, L. R., and Pickett, M. J., *J. Gen. Physiol.*, **24**, 33–44 (1940).

QUINOLINIC ACID ACCUMULATION IN THE CONVERSION OF 3-HYDROXYANTHRANILIC ACID TO NIACIN IN NEUROSPORA*

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3-Hydroxyánthranilic acid has been shown to serve as a natural precursor of niacin in Neurospora.[1,2] Little information, however, has been obtained concerning the mechanism whereby the aromatic ring of 3-hydroxyanthranilic acid is converted to the pyridine ring of niacin. The present paper will deal with this latter problem.

Singal, *et al.*,[3] have reported that the administration of tryptophan to rats results in the urinary excretion of a compound possessing niacin activity for *Lactobacillus arabinosus* after autoclaving in acid solution. This compound has recently been isolated and identified by Henderson[4] as quinolinic acid (2,3-dicarboxypyridine). These data suggest that quinolinic acid plays a rôle in the biosynthesis of niacin. Quinolinic acid had been tested earlier on certain mutant strains of Neurospora requiring niacin for growth, and found to be inactive.[1,2] However, it seemed desirable to determine whether this compound might not be accumulated by niacin-requiring mutant strains, and have escaped attention because of its inactivity. One mutant strain has been found which accumulates this compound as a result of a genetic block.

Experimental.—The various mutant strains of Neuorspora used in this investigation, together with the compounds known to support their growth are listed in table 1. The basal medium used throughout is the customary

TABLE 1

GROWTH OF VARIOUS STRAINS OF NEUROSPORA

STRAIN NO.	GROWTH ON MINIMAL MEDIUM PLUS: NO ADDITION	NIACIN	QUINOLINIC ACID	3-OH-ANTHRANILIC ACID	KYNURENINE	TRYPTOPHAN	ANTHRANILIC ACID
3416	−	+	−	−	−	−	−
4540	−	+	−	−	−	−	−
Y31881	−	+	−	+	−	−	−
39401	−	+	−	+	+	+	−
75001	−	−	−	−	+	+	+
10575	−	−	−	−	−	+	−
Wild type	+	+	+	+	+	+	+

Neurospora minimal,[5] and growth tests were made for 72 hours at 25°C. in 125-cc. Erlenmeyer flasks containing 20 cc. of medium, unless otherwise stated. The *L. arabinosus* assay for niacin is that described by the Association of Vitamin Chemists.[6]

Of the mutant strains which require niacin for growth, only strains 4540 and 3416 are known for which some known substance cannot replace the niacin requirement. These two strains are both genetically and biochemically different. Crosses between 3416 and 4540 give wild-type progeny,[7] and strain 4540 has been shown to accumulate 3-hydroxyanthranilic acid,[1] while strain 3416 does not. Of the various niacin-requiring mutant strains, therefore, only strain 3416 might reasonably be suspected of quinolinic acid accumulation since the other strains are genetically blocked prior to the utilization of 3-hydroxynathranilic acid.

Extensive tests of strain 3416 culture filtrates failed to show significant accumulation of an intermediate which would support the growth of any other niacin-requiring strains. Such culture filtrates, however, when autoclaved for 15 minutes at 15-lb. pressure in 1 *N* HCl had marked niacin activity by Lactobacillus assay (see table 2) whereas similar filtrates not so treated had little niacin activity. For the unequivocal demonstration of the presence of quinolinic acid in culture filtrates of strain 3416, sufficient culture filtrate was prepared to permit the isolation of this dicarboxy acid.

TABLE 2

QUINOLINIC ACID ACCUMULATION BY STRAIN 3416

3416 CULTURE FILTRATE TESTED IN ML.	γ NIACIN		γ QUINOLINIC ACID CONVERTED TO NIACIN PER ML. FILTRATE	γ QUINOLINIC ACID PER ML. FILTRATE
	BEFORE ACID AUTOCLAVING	AFTER ACID AUTOCLAVING		
0.03	0.02	0.3	9.3	78
0.003	0	0.03	10	83
Quinolinic acid 3.3 γ	0	0.4 (12% conversion)	..	..

The method of isolation used was a modification of the method outlined by Henderson.[4] Nineteen liters of basal medium contained in a 5-gal. Pyrex bottle, supplemented with 10 mg. of niacin, were inoculated with strain 3416 and the strain grown with forced aeration for seven days at 25°C. The mycelium was removed by straining through cheesecloth, and the culture filtrate concentrated to ca. 2 liters. This concentrate was filtered and acidified to pH1, and shaken with 100 g. of Norit A. After filtration the norit was treated with dilute ammonium hydroxide. This eluate was evaporated to dryness, and the residue treated with about 30 cc. of methanol slightly acidified with HCl. The entire methanol-soluble fraction was then put on a single sheet of Schleicher and Schuell paper No. 470-A (0.025 inch thick) by the method of Yanofsky, *et al.*,[8] and developed as an ascending chromatogram using butanol saturated with water containing 8% formic acid as the developing solvent. Pure quinolinic acid was run with the same solvent to determine its Rf value. The quinolinic acid

band was cut from the dried sheet, eluted with dilute NH_4OH in a Waring blendor and the resulting eluate evaporated to dryness. Quinolinic acid was then crystallized from slightly acidified dilute ethanol. Recrystallization gave a material which began to sublime at ca. 165°, and melted with decomposition and gas evolution at 188–190°. When this material was mixed with authentic quinolinic acid no depression in the melting point was observed. Figure 1 gives a comparison of the absorption spectra of the

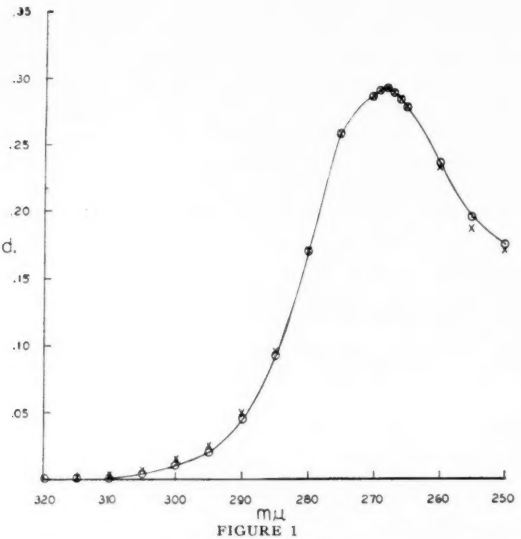

FIGURE 1

Comparison of absorption spectra of quinolinic acid isolated from strain 3416 culture filtrates, and authentic material. ⊙—⊙ quinolinic acid. x—x isolated material.

isolated material and authentic quinolinic acid in 0.1 N HCl. The crystalline material was inactive for growth of *L. arabinosus*, but showed conversion to niacin after autoclaving in acid solution. The isolated material, therefore, is identified as 2,3-dicarboxypyridine.

In evaluating the significance of these observations, a second point must be clarified. The accumulation of quinolinic acid appears to be associated with niacin synthesis in rats and dogs. Is this accumulation characteristic of the synthesis of niacin by Neurospora, or is it a unique property of strain 3416? A variety of strains were, therefore, tested for quinolinic acid accumulation as shown in table 3. Quinolinic acid accumulation is clearly asso-

ciated only with strain 3416, and may therefore be considered as peculiar to that particular genetic block.

Discussion.—The demonstration by Singal, *et al.*,[3] and by Henderson[4] that quinolinic acid results from the administration of large amounts of tryptophan to rats suggests that quinolinic acid is involved in the conversion of tryptophan to niacin. Henderson[4] further showed that quinolinic acid resulted from the intraperitoneal injection of 3-hydroxyanthranilic acid,

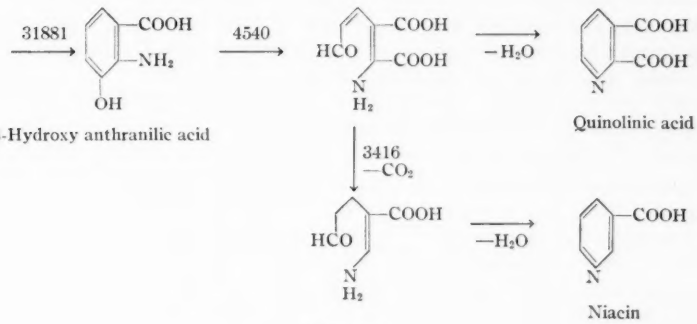

FIGURE 2

Proposed scheme for the conversion of 3-hydroxyanthranilic acid to niacin and quinolinic acid.

TABLE 3

QUINOLINIC ACID ACCUMULATION BY STRAINS OF NEUROSPORA*

STRAIN NO.	γ NIACIN PER CC. FILTRATE	
	BEFORE ACID AUTOCLAVING	AFTER ACID AUTOCLAVING
3416	0	9.3
4540	0.4	0.4
Y31881	0.4	0.4
39401	0.3	0.9
75001	0.4	0.4
10575	0.0	0.0
Wild type	0.0	0.0

* Not corrected for per cent conversion.

suggesting that quinolinic acid serves as an intermediate in the conversion of 3-hydroxyanthranilic acid to niacin.

The observations in Neurospora are in substantial agreement with those made on the rat, with the exception that quinolinic acid is inactive even for those mutants which are known to be capable of converting 3-hydroxy anthranilic acid to niacin. This inactivity of quinolinic acid in turn suggests a mechanism of conversion shown in figure 2. This scheme suggests

that 3-hydroxyanthranilic acid serves as the precursor both of niacin and quinolinic acid. That 3-hydroxyanthranilic acid serves as a precursor of niacin has been previously demonstrated.[1, 2] That 3-hydroxy anthranilic acid also serves as a precursor of quinolinic acid may be argued from the fact that quinolinic acid is accumulated by a strain which cannot either utilize 3-hydroxyanthranilic acid (in place of niacin) or accumulate it. All other niacin-requiring mutant strains of Neurospora possess one or the other of these two abilities. Quinolinic acid accumulation must, therefore, result from a block interposed in the conversion of 3-hydroxyanthranilic acid to niacin. Further evidence in this connection is the fact that 3-hydroxyanthranilic acid increases quinolinic acid accumulation by strain 3416, and that in preliminary experiments with washed resting 3416 mycelium, quinolinic acid results from the addition of 3-hydroxyanthranilic acid. The scheme given in figure 2 further suggests cleavage in the 3,4 position with the formation of a dicarboxy aliphatic amino aldehyde. No evidence for this step is available other than the general logic of the entire scheme and the fact that 3416 does not accumulate an active substance but does accumulate quinolinic acid. The proposed intermediate might be expected to undergo ring closure very readily. In view of the inactivity of quinolinic acid, however, ring closure must normally be preceded by decarboxylation in the formation of niacin. If decarboxylation cannot occur, as the result of a genetic block, ring closure would result in quinolinic acid formation. It should be pointed out that the validity of such a scheme rests upon the demonstration of the proposed intermediates. The proposed scheme, however, is in agreement with present data and gives, perhaps, a clearer insight into the general problem of the biological formation of pyridine ring compounds.

Summary.—Quinolinic acid has been isolated from culture filtrates of a mutant strain of Neurospora requiring niacin for growth. Quinolinic acid is inactive in supporting growth of niacin-requiring strains. The observation that an inactive compound, quinolinic acid, is accumulated as the result of a genetic block, suggests a mechanism for the conversion of the benzene ring of 3-hydroxyanthranilic acid to the pyridine ring of niacin.

Quinolinic acid has been found to have slight activity for strains 4540, 39401 and 31881. The activity of this acid, after correction for per cent dissociation ($pk_1 = 3.0$, $pk_2 = 5.0$) is at most one-fifth that of niacin. In view of this low activity, and the fact that the precursor of quinolinic acid, 3-hydroxyanthranilic acid, is as active as niacin, the authors feel that the scheme presented in figure 2 is still the most logical.

* These investigations were supported in part by the Williams-Waterman Fund for the combat of dietary diseases, and in part by the Rockefeller Foundation.

[1] Bonner, D., PROC. NATL. ACAD. SCI., **34,** 5 (1948).

[2] Mitchell, H. K., and Nyc, J. C., *Ibid.*, **34,** 1 (1948).

[3] Singal, S. A., Briggs, A. P., and Hankes, L. V., *Proc. Soc. Exptl. Biol. Med.*, **70**, 26 (1949).

[4] Henderson, L. M., *J. Biol. Chem.*, **178**, 1005 (1949).

[5] Beadle, G. W., and Tatum, E. L., *Am. J. Bot.*, **32**, 678 (1945).

[6] The Association of Vitamin Chemists, Methods of Vitamin Assay. Interscience Press, New York (1947).

[7] Bonner, D., and Beadle, G. W., *Arch. Biochem.*, **11**, 319 (1946).

[8] Yanofsky, C., Wasserman, E., and Bonner, D., *Science*, in press.

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*THE RÔLE OF ORGANIC PEROXIDES IN THE INDUCTION OF MUTATIONS**

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The discovery by Wyss, Stone, and Clark[1] that bacteria grown on a substrate recently exposed to ultra-violet light are subject to high mutation rates shows clearly that some meta-stable chemical substance, probably of no great complexity, is an intermediate in at least a part of the mutagenic action of ultra-violet light. It was supposed that hydrogen peroxide might be responsible for these results, but subsequent work has shown that this cannot be the whole explanation.[2] However, organic peroxides are known to be formed by the action of ultra-violet light on many compounds and such peroxides might very well be the intermediate agents producing the substrate irradiation effect.

The process by which organic compounds, especially ethers, olefins and aldehydes, form peroxides simply on contact with molecular oxygen is not wholly understood. In many simple cases, however, a chain reaction of the sort pictured below appears to be involved.

$$\begin{aligned} \text{R—H} &\longrightarrow \text{R—} + \text{H} \\ \text{R—} + \text{O}_2 &\longrightarrow \text{R—OO—} \\ \text{R—OO—} + \text{R—H} &\longrightarrow \text{R—OO—H} + \text{R—} \end{aligned}$$

The peroxide-forming process is catalyzed by ultra-violet light[3] which, presumably, supplies the energy for breaking a carbon-hydrogen bond in the first step.

The hypothesis that organic peroxides play an essential rôle in the mutagenic action of ultra-violet light has been under investigation in this laboratory for some time. One result of this work, and the subject of the

present writing, is the discovery that many simple organic peroxides increase mutation rates.

Testing Procedure.—The organism used for detecting and comparing mutagenic agents has been an adenineless, colonial strain of *Neurospora crassa*, the double mutant 70007-38701. The adenineless character in this strain is subject to a low spontaneous rate of reversion to adenine independence. These occurrences are probably back mutations at the adenineless locus, but this has not been demonstrated in the present study. The colonial character, introduced into the strain to permit plate counts, appears to be quite stable. Following essentially a method described by Giles and Lederberg[4] and by Westergaard and Mitchell,[5] conidial suspensions were exposed to various peroxides and the effects calculated from the fraction of these spores that gave rise to colonies on adenine-free medium. Since as many as 2×10^8 spores may be conveniently treated in a single experiment, this method is capable of detecting very weak mutagenic activity.

In a typical experiment a thoroughly mixed suspension of two-day old spores was divided into four portions, centrifuged and decanted. Two portions were retained as controls and the other two were re-suspended in an aqueous solution of the peroxide and allowed to stand at room temperature for 30 minutes. After washing to remove the treating solution, the concentration of conidia in each centrifuge tube was determined with a hemocytometer, and dilution platings on adenine-supplemented medium were made from each sample to indicate conidial viability and percentage mortality. Finally, the suspensions were spread on a series of adenine-free plates and incubated at 25°C. Mutants formed distinct colonies that could be counted during the third or fourth day after treatment.

Mutation rates were calculated by dividing the number of mutants counted by the number of spores plated. By subtracting from the mutation rate shown by spores subjected to a particular treatment the spontaneous rate shown by untreated spores from the same spore batch there was obtained the quantity termed the "induced mutation rate." It is not practical to determine an average value for the fraction of untreated spores that produce adenine-independent colonies and to use such a fixed figure for correcting the observed mutation rates. Although the spontaneous rate is lower than 0.8×10^{-7} in more than 75% of the spore batches, occasionally this rate is very high (e.g., 13×10^{-7}), presumably as a result of an early mutation in the culture from which the spores were obtained. The same problem has been described by Delbrück[6] in connection with mutations in bacteria.

It appears that the largest numbers of mutants are obtained with treatments that kill 60 to 80% of the spores. Mutation rates based on the numbers of spores surviving the treatment might increase up to very high

mortalities but in the present investigation more consistent values have been obtained for mutation rates based on the number of spores treated. Accordingly, treatments producing the greatest actual numbers of viable mutants have been sought.

A danger in working with very high mortalities is the occasional appearance of numbers of adenineless colonies, apparently sustained by adenine released to the medium from dead spores. Such false mutants can usually be distinguished by their frail appearance and their failure to develop beyond an early stage, and always by their inability to grow when transferred to minimal medium.

Demonstration of the Mutagenic Action of Peroxides.—Table 1 illustrates the mutagenic action of *tert*-butyl hydroperoxide at various concentrations. The data are taken from typical experiments selected from an extensive study of this material. The table is intended to show the effect of concentration of the agent and to give a rough idea of the reproducibility of the results at a particular concentration. The first objective of this work has been to show that peroxides induce mutations and the values obtained for induced mutation rates are only approximate. Variations in the conditions of treatment, especially temperature, may account in part for the irregularities in the results.

TABLE 1

MUTAGENIC ACTION OF *tert*-BUTYL HYDROPEROXIDE

(Treatment: 30 minutes exposure to an aqueous solution of *tert*-butyl hydroperoxide at the indicated concentration)

CONCENTRATION, MOLES PER LITER	SPORES TREATED, MILLIONS	MUTANTS	MUTATION OBSERVED	RATES × 10^7 INDUCED	MORTALITY, %
0.004	48	6	1.2	0.5	5
0.010	96	51	5.3	2.5	42
0.089	70	106	15.1	15.0	29
0.089	72	140	19.3	19.2	38
0.089	40	51	12.8	10.5	80
0.089	29	21	7.2	7.1	91
0.11	32	6	1.9	1.8	85
0.27	38	0	0.0	0.0	100

In Table 2 averages of the results of many experiments with six different peroxides are presented. The concentrations of the respective agents, shown in this table, are the ones which have given the highest mutation rates. For comparison the effects of four established mutagenic agents have been included and also the results of experiments with four mildly toxic substances which had no definite effect on mutation rates. It might be noted that two of the last named group, phenol and formaldehyde, have been reported to have a weak mutagenic action in experiments with Drosophila.[7, 8]

The active principles in the mixtures of hydrogen peroxide with formaldehyde and acetone may be, respectively, HO—CH_2—OO—CH_2—OH and HO—$C(CH_3)_2$—OO—H. Hydroxymethyl *tert*-butyl peroxide, derived similarly from the interaction of *tert*-butyl hydroperoxide and formaldehyde, is a well-defined compound.[9] The peroxide derived from diisopropyl ether was obtained by extracting old samples of the ether with water and freeing the extract of volatile material by passing an air stream through it.

TABLE 2

MUTAGENIC ACTION OF VARIOUS AGENTS

(Treatment: 30 minutes exposure to indicated aqueous solutions)

AGENT	CONCENTRATION, MOLES PER LITER	TOTAL SPORES, MILLIONS	INDUCED MUTATION RATE × 10^5	MORTALITY, %
Hydrogen peroxide	0.21	1200	1.8	40
tert-Butyl hydroperoxide	0.089	211	14.5	50
Hydroxymethyl *tert*-butyl peroxide	0.089	270	16.3	68
Peroxide derived from diisopropyl ether	0.15	146	23.0	61
Hydrogen peroxide and formaldehyde	0.022 (0.033 in CH_2O)	464	8.8	77
Hydrogen peroxide and acetone	0.21 (1.36 in $(CH_3)_2CO$)	96	10.4	32
X-rays	*	61	15.8	37
Ultra-violet light	†	418	46.0	32
Bis(β-chloroethyl)sulfide	0.0002	103	4.0	70
Bis(β-chloroethyl)methylamine	0.004	279	3.6	85
Phenol	0.077	465	−0.4	87
Formaldehyde	0.0244	251	0.5	73
Potassium permanganate	0.00052	383	0.0	35
tert-Butyl alcohol	1.75	275	−0.3	84

* Approximately 30,000 r units.

† Exposure of an aqueous suspension with agitation for 75 seconds to 30-watt General Electric germicidal lamp at a distance of 10 cm. The incident ultra-violet energy, nearly all in the vicinity of 2537 A. U., was 6000 ergs/mm.²/min.

The peroxide concentration in the resulting solution was determined iodometrically.

Mustard gas, *bis*(β-chloroethyl)sulfide, and the nitrogen mustard, *bis*(β-chloroethyl)methylamine, were used in borate (pH 8) and acetate (pH 5) buffers, respectively. The solution of the sulfur compound contained, in addition, 1.36 moles per liter of acetone to increase its solubility.

In presenting this demonstration of the mutagenic action of peroxides it is assumed that the adenine-independent colonies appear as the result of true mutations. The action of the peroxides certainly produces a heritable change since adenine independence of the reverted strains persists through repeated subcultures on an adenine-free medium. It has been the general experience of other workers that non-genetic reversions of Neurospora mutants, which occur spontaneously in some strains, do not persist through subcultures. This, together with the observation that known mutagenic agents (radiations, mustard gas) produce effects similar to those of the peroxides, constitutes presumptive evidence for the view that the action of the peroxides is mutational. It is recognized, however, that the results of genetic crosses will constitute the only proof that gene mutations are involved. A genetic study directed toward this end is now in progress and will be reported in a later communication.

There can be little doubt that the peroxide treatment *induces* the presumed mutations. Selection cannot account for the results since the spores are not growing during the treatment and since it is an increase in the actual number of adenine-independent colonies that is observed and not merely a higher ratio of the number of such colonies to the number of surviving spores.

Conceivably, certain nuclei (the conidiospores are multinucleate) possess a latent capacity for adenine synthesis that is only made manifest by the peroxide treatment. However, there is no precedent for such a phenomenon. This question as well as the question of the mutational nature of the peroxide-induced changes will be resolved by the current genetic studies mentioned above.

Finally, it should be noted that the experimental method used here is of a rather special sort. In all probability, only the back mutation of a single gene is involved. It will be important to confirm these findings by different techniques and in different organisms.

Conclusions.—The foregoing evidence that organic peroxides are capable of producing mutations lends substantial support to the idea that ultraviolet light produces mutations by forming such compounds in irradiated media and in cells. A demonstration that this is the mechanism of the substrate-irradiation effect may eventually be obtained by correlating the mutagenic action of irradiated material with peroxide content.

The facts that so few mutation-inducing agents are known and that the effects of those that are known are in large measure similar suggest that they have a common mode of action. Accordingly, the possibility that peroxides are in some way involved in the action of x-rays and of the mustards is currently under investigation. Possibly compounds of the mustard group or certain products of their interaction with organic compounds are especially liable to peroxide formation.

By determining just what feature of the chemistry of peroxides is responsible for their mutagenic action one might hope to shed light on the nature of the mutation process. It seems unlikely that this action is simply related to oxidizing power, since oxidizing agents are common and organic peroxides are not especially effective ones. Of more interest is the characteristic decomposition of peroxides by which free radicals are produced. If this is the essence of peroxide action non-peroxidic free radical sources (e.g., diazomethane) should show similar effects. It should be noted that irradiation of a cell could produce free radicals directly as well as by peroxide formation.

Besides affording a basis for speculation on the nature of the mutation process, the discovery of the mutation-inducing power of organic peroxides substantially increases the number of known mutagenic agents. Organic peroxides of widely varied structure can be prepared. It will be of interest to compare the action of these various agents on different genes and to search for agents having pronounced effects on particular genes.

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† DuPont Predoctoral Fellow in Chemistry 1948–1949.
‡ The authors wish to thank Professor Norman H. Horowitz for valuable assistance and advice.

[1] Wyss, O., Stone, W. S., and Clark, J. B., *J. Bact.*, **54,** 767–772 (1947).
[2] Wyss, O., Clark, J. B., Haas, F., and Stone, W. S., *J. Bact.*, **56,** 51 (1948).
[3] Milas, N. H., *J. Am. Chem. Soc.*, **53,** 221–233 (1931).
[4] Giles, N. H., Jr., and Lederberg, E. Z., *Am. J. Bot.*, **35,** 150–157 (1948).
[5] In an unpublished investigation this method for detecting chemical mutagens was developed by M. Westergaard and H. K. Mitchell to whom the authors are also indebted for the stock.
[6] Delbrück, M., *Ann. Mo. Bot. Gard.*, **32,** 223–233 (1945).
[7] Hadorn, E., Rosin, S., and Bertani, D., *Proc. 8th Int. Congr. Gen.*, 256–266 (1949).
[8] Rapoport, I. A., *Acad. Sci. U.R.S.S.*, **54,** 65–67 (1946).
[9] Dickey, F. H., Rust, F. F., and Vaughan, W. S., *J. Am. Chem. Soc.*, **71,** 1432–1434 (1949).

CROSSING-OVER BETWEEN ALLELES AT THE LOZENGE LOCUS IN DROSOPHILA MELANOGASTER

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It has been reported that females of *Drosophila melanogaster* having one *X*-chromosome containing the lozenge allele glossy (lz^g) and the other *X*-chromosome the lozenge allele spectacle (lz^s), crossed to either lz^g or lz^s males, occasionally produce individuals wild type (lz^+) in appearance.[1,2]

The occurrence of wild-type progeny from this cross is unexpected since all the progeny of both sexes should have the phenotype of either lz^s or lz^g. Furthermore, it has been noted that the production of the non-mutant X-chromosome has resulted when crossing over has occurred in the vicinity of the lozenge locus. These results suggest that either the lz^+ chromosome occurred as a result of unequal crossing over between lz^g and lz^s in the same manner as Bar in *D. melanogaster*,[3] or that lz^g and lz^s are in fact not alleles in the usual sense but represent two closely linked loci, such that an ordinary crossover between them would yield an lz^+ chromosome.

In view of the information already obtained, experiments were set up to determine (1) whether the phenomenon of crossing over between lz^g and $lz^s \rightarrow lz^+$ was peculiar to these lozenge alleles, or whether it occurred between other lozenge mutants as well; (2) whether the crossing over involved is equal or unequal; and (3) what is the nature of the complementary crossover X-chromosome, presumably bearing two lozenge mutants.

TABLE 1

RECOMBINATION RESULTS FROM ♀ ♀ HETEROZYGOUS FOR TWO DIFFERENT LOZENGE ALLELES CROSSED TO ♂ ♂ *In(1)dl 49, v lz*^{g}

GENOTYPE OF ♀ ♀	TOTAL F_1 ♂ ♂ AND ♀ ♀	lz^+ ♂ ♂ AND ♀ ♀	lz^g-LIKE ♂ ♂	PER CENT CROSSING-OVER*
$lz^{BS}/sn^3\ lz^{46}\ ras^4\ v$	20,554	9†	5‡	0.09
$lz^{BS}/ec\ ct^6\ lz^g\ v\ f$	16,255	13§	5‖	0.14
$lz^{46}/ec\ ct^6\ lz^g\ v\ f$	16,098	4§	3‖	0.06

* See text for method of calculation.
† All carried sn^3. ‡ All carried $ras^4\ v$.
§ All carried ct^6. ‖ All carried v.

Three lozenge alleles were used in attempting to answer these questions: lz^{BS}, which occurred as a result of X-irradiation in $T(1:4)B^s$ and which has been separated from the translocation; lz^{46}, which is of spontaneous origin, and lz^g, which is the same mutant referred to above except that it has been extracted from *In(1)dl 49* where it originally occurred as a result of X-irradiation. All these mutants are typical lozenge alleles, recessive to wild type, and phenotypically characterized by derangement of the eye facets, reduction in the eye pigment and female infertility related to the absence of spermathecae.[2, 4, 5] All possible heterozygotes of the three alleles (e.g., lz^{BS}/lz^{46}) are lozenge in phenotype and difficult to separate phenotypically from either homozygote. Compounds among the three mutants were made and back-crossed to males of the genotype *In(1)dl 49, v* lz^g. The results are listed in table 1.

From the results tabulated in table 1, it may be noted that in the cross of females $lz^{BS}/sn^3\ lz^{46}\ ras^4\ v\ X$ males *In(1)dl 49, v* lz^g (where sn^3 lies 6.7 units to the left and ras^4 and v lie 5.1 and 5.3 units, respectively, to the right of

lozenge) two unexpected classes of offspring occur. One class is wild type in appearance and carries the marker gene sn^3. These individuals are phenotypically identical to the lz^+ flies recovered from females lz^g/lz^s. The second class comprises individuals which carry the marker genes ras^4 and v and are phenotypically completely separable from both lz^{BS} and lz^{46}, and are in fact identical to lz^s in phenotype. While lz^+ being dominant to both lz^{BS} and lz^{46} was recovered in both males and females, the lz^s-like individuals were males only. The failure to recover the lz^s-like chromosome in females stems from its phenotypic inseparability when compounded to lz^{g3} consequently only one-half the expected number were found. This accounts for the discrepancy between the lz^+ and lz^s-like individuals in table 1 which theoretically should be equal in frequency.

It may be reasoned that the lz^+ flies carry an X-chromosome which as the result of crossing over carries wild-type alleles for both lz^{BS} and lz^{46}, while the lz^s-like flies represent the complementary crossover with lz^{BS} and lz^{46} on the same X-chromosome. If this is the case, then it should be possible to recover both lz^{BS} and lz^{46} separately in the progeny of females of the genotype lz^+/lz^s-like. That this is the case may be determined from the following results. Females were constituted having one X-chromosome carrying lz^s-like (presumably lz^{BS} and lz^{46} together) plus the marker genes ras^4 and v, and the other an lz^+ X-chromosome (derived from crossing over between lz^{BS} and lz^{46}) bearing the marker gene sn^3. Among 12,900 male offspring from these females, three males of the genotype $sn^3\ lz^{46}\ ras^4\ v$ and four males lz^{BS} were recovered. These results support the hypothesis that the lz^+ chromosome carries wild-type lozenge alleles and the lz^s-like chromosome carries both lz^{BS} and lz^{46} and therefore might be designated as $lz^{BS,\ 46}$. That the lz^+ chromosome does contain only wild-type lozenge alleles is supported by one additional experimental finding. Females were constituted having one X-chromosome carrying $lz^{BS,\ 46}\ ras^4\ v$ and one chromosome derived from the Canton-S wild-type stock and carrying the marker genes sn^3 and v. Among 9100 male offspring of these females, one male $sn^3\ lz^{46}\ ras^4\ v$ and three males $lz^{BS}\ v$ were recovered, thereby confirming the identity of the lz^+ chromosome with a wild-type X-chromosome and therefore as bearing only wild-type lozenge alleles.

The results reported thus far indicate that crossing-over is not peculiar to the alleles lz^g and lz^s since it occurs also between other alleles, lz^{BS} and lz^{46}. They indicate also that the crossing-over between alleles is equal and not unequal as in the case of Bar, for the following reasons. First, in no instance among many thousands of flies examined have unexpected individuals (lz^+ or lz^s-like) been observed from females homozygous for lz^{BS} or lz^{46}. Second, the crossover individuals resulting from females lz^{BS}/lz^{46} are directed in so far as the marker genes are concerned. All lz^+ individuals carry the marker sn^3 while all $lz^{BS,\ 46}$ individuals carry ras^4 and v. If un-

equal crossing-over were involved then one would expect to get lz^+ flies carrying either sn^3 or $ras^4\,v$, and not always and only sn^3 as noted here. Similarly, $lz^{BS,\ 46}$ flies would be expected to carry either sn^3 or ras^4 and v, but not solely ras^4 and v. Consequently, it seems reasonable to conclude that regular crossing-over has occurred between two closely linked genes, with lz^{BS} located just to the left of lz^{46}.

It may be noted in the cross of females $lz^{BS}/ec\ ct^6\ lz^g\ v\ f$ *X* *In(1)dl 49*, $v\ lz^g$ males (where *ec* and ct^6 lie 22.2 and 7.7 units, respectively, to the left and v and f lie 5.3 and 29 units, respectively, to the right of lozenge) that here too unexpected progeny of two types were obtained (cf. table 1). The lz^+ group is phenotypically identical to the lz^+ flies recovered from females lz^{BS}/lz^{46}, while the complementary crossover individuals, presumably carrying both lz^{BS} and lz^g on the same chromosome, are phenotypically inseparable from flies $lz^{BS,\ 46}$. As in the case of lz^+ flies from females lz^{BS}/lz^{46}, the lz^+ flies derived from females lz^{BS}/lz^g all carry the left marker gene proximal to lozenge, namely ct^6. Similarly, the flies presumably $lz^{BS,\ g}$ carry the marker proximal to lozenge on the right, namely v.

These results indicate that the mutant lz^g lies to the right of lz^{BS}. That lz^g also lies to the right of the lz^{46} and represents a third locus in the lozenge group of mutants may be readily deduced from the results obtained when females $lz^{46}/ec\ ct^6\ lz^g\ v\ f$ are crossed to males *In(1)dl 49*, $v\ lz^g$. It can be seen that once again two unexpected classes of progeny are obtained (cf. table 1). The lz^+ individuals are phenotypically identical to the lz^+ flies referred to previously and in this cross all carry the marker gene ct^6. The second class of unexpected individuals may again be described as lz^s-like and presumably carry both lz^{46} and lz^g on the same chromosome. In addition all such flies recovered carry the marker gene v. On the basis of the directed distribution of the marker genes to the lz^+ and the lz^s-like individuals, it seems logical to represent lz^g as lying to the right of lz^{46}.

Discussion.—These observations may be fitted into a scheme which specifies that the lozenge allelic series is genetically divisible into at least three closely linked loci in the order of lz^{BS}–lz^{46}–lz^g, proceeding from left to right along the *X*-chromosome. The genetic interval separating lz^{BS} and lz^{46} is of the order of 0.09% crossing-over, and the interval separating lz^{46} and lz^g is of the order of 0.06% crossing-over. (The intervals have been calculated after first doubling the crossovers giving two lozenge genes in the same *X*-chromosome since, as noted previously, only one-half of these could be recovered.) If this interpretation is correct, then a wild-type *X*-chromosome may be represented as carrying three wild-type genes each allelic to one of the three lozenge mutant genes. With this as a basis, the *X*-chromosome bearing each of the mutant genes may be represented genotypically as follows: $lz^{BS}\ +\ +$; $lz^{46}\ +$; and $+\ +\ lz^g$.

Phenotypic differences have been noted with combinations of any two

of the lozenge mutants depending on whether the mutants ard located together on the same X-chromosome or separately on homologous X-chromosomes. Using the genotypic notation proposed, the following combinations of mutant genes have been compared and their phenotypes recorded after each genotype:

lz^{BS} + +/+ lz^{46} + (mutant); lz^{BS} lz^{46} +/+ + + (wild type)
+ lz^{46} +/+ + lz^{g} (mutant); + lz^{46} lz^{g}/+ + + (wild type)
lz^{BS} + +/+ + lz^{g} (mutant); lz^{BS} + lz^{g}/+ + + (wild type)

It may be noted that in all cases the number of genes, mutant and wild type, is identical. Furthermore in all cases each mutant gene is balanced by a wild-type allele on the homologous X-chromosome. Yet when a lozenge mutant is present on each homologous X-chromosome, the wild-type alleles behave as recessive genes (or the lozenge mutants act as dominant genes), but when the same lozenge mutants are located together on the X-chromosome (even when separated by a wild-type allele) and the homologous X-chromosome carries only wild-type alleles, the wild-type alleles together act as dominant genes (or the lozenge mutants behave as recessives). These dominance relationships may be interpreted as being the result of a position effect. Similar observations have been made in the case of the interactions of the pseudoalleles Star and asteroid in *D. melanogaster*.[6]

A further conclusion which may be drawn in the light of additional information (unpublished) is that the three lozenge loci represent a reduplication of essentially identical genetic material. A number of other lozenge alleles (10 of the 14 which we possess) have been analyzed to determine to which locus they may be assigned.[7] While this analysis is incomplete, the data obtained thus far permit three generalizations which support this conclusion. In the first place, it is clear that the mutants at each locus possess the same array of phenotypic effects, vis., alteration of eye color and structure, infertility of females, etc. Secondly, if the mutants are classified quantitatively (e.g., with respect to the amount of red eye pigment formed), it can be seen that they are distributed at random to the three loci. Lastly, there appears to be no correlation between the mode of origin of the various mutants (i.e., spontaneous, x-ray induced) and their position. These findings are somewhat at variance with the observations made in cotton where three closely linked loci determining anthocyanin pigmentation have been described.[8, 9] In the latter case, although the three loci are in some instances interdependent, it cannot be said that all three possess the same array of phenotypic effects, suggesting that these loci represent three closely linked though not necessarily identical genes.

In addition to the cases cited, additional occurrences of crossing-over between what are apparently alleles have been observed in maize[10] and Drosophila,[11] and possibly in the case of the Brachy series in mice.[12] Just how these cases relate to the lozenge series is not at the present time com-

pletely clear, but it may be that all represent further examples of duplication or reduplication of the same genic material. It is not possible at present to extrapolate the data of the lozenge series to other allelic series. However, they do not justify the conclusion that all multiple allelic series represent cases of multiple loci, for the simplest explanation for the situation within the lozenge complex itself would appear to be that of three closely linked multiple allelic series.

Summary.—1. Crossing-over has been observed between three sex-linked, recessive lozenge alleles, lz^{BS}, lz^{46}, lz^{9} in *D. melanogaster*.

2. From females heterozygous for any two of the mutants, wild type X-chromosomes and X-chromosomes bearing two lozenge mutants have been recovered.

3. On the basis of the crossover results, the lozenge allelic series may be subdivided into three closely linked loci.

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[1] Oliver, C. P., PROC. NATL. ACAD. SCI., **26,** 452–454 (1940).
[2] Oliver, C. P., and Green, M. M., *Genetics*, **29,** 331–347 (1944).
[3] Sturtevant, A. H., *Ibid.*, **10,** 117–147 (1925).
[4] Oliver, C. P., *U. Texas Publ.*, **4720,** 167–184 (1947).
[5] Green, M. M., *Am. Natl.*, **82,** 188–192 (1948).
[6] Lewis, E. B., *Genetics*, **30,** 137–166 (1945).
[7] Green, M. M., and Green, K. C., unpublished.
[8] Stephens, S. G., *Genetics*, **33,** 119–214 (1948).
[9] Yu, Chi Pao, and Chang, Te Sung, *J. Genetics*, **49,** 46–56 (1948).
[10] Laughnan, J. R., PROC. NATL. ACAD. SCI., **35,** 167–178 (1949).
[11] Lewis, E. B., *Genetics*, **33,** 113 (1948).
[12] Dunn, L. C., and Caspari, E., *Ibid.*, **30,** 543–568 (1945).

EXPERIMENTS ON LIGHT-REACTIVATION OF ULTRA-VIOLET INACTIVATED BACTERIA

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Many types of microorganisms are killed by ultra-violet light and the number of survivors falls off with increasing dose. A. Kelner[1] reported recently on his discovery that, if exposure to ultra-violet light is followed by exposure to visible light, the number of survivors is very much larger. A similar discovery was reported by R. Dulbecco[2] for bacteriophage.

We investigated this phenomenon of light-reactivation of ultra-violet inactivated bacteria and found some very simple regularities.

In our experiments we used a strain of coli, B/r, originally isolated by E. M. Witkin.[3] Cultures were grown in a lactate-ammonium phosphate medium under aeration to between 5×10^7 and 10^8 bacteria per cc., then transferred into saline, and incubated under aeration at 37°C. from 14 to 18 hours. The cultures were then placed in the icebox, kept at about 6°C. and used for experiments over a period of about one week.

Figure 1 shows in semilogarithmic plot the result of one series of experiments. Eight other such series were conducted with similar results.

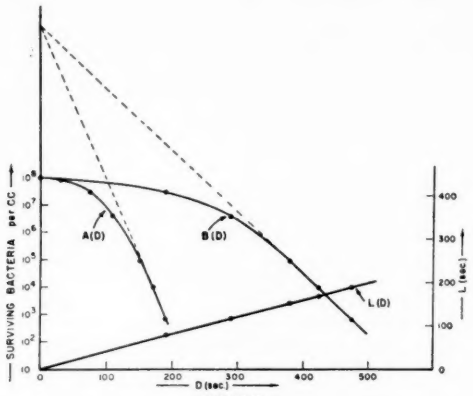

FIGURE 1

Experiment of May 11, 1949. $A(D)$, left scale, gives in semi-log plot the number of survivors as function of ultra-violet dose, D, in seconds of exposure to 15-watt germicidal lamp at 50 cm. distance (no light reactivation). $B(D)$, left scale, gives in semi-log plot the number of survivors as function of ultra-violet dose, D, with light reactivation by 1-hour exposure to light of 1000-watt projection lamp at 8 inches distance. $L(D)$, right scale, gives in linear coordinates L as function of D satisfying equation $B(D) = A(L)$.

In these series of experiments the survivor curves, $A(D)$, were obtained in the following manner: The bacteria were exposed in saline to different ultra-violet doses, D, characterized by the number of seconds of exposure, then plated on nutrient broth agar, incubated, and the survivors determined by colony count. A 15-watt germicidal lamp was used as the source of ultra-violet radiation with the mercury line at 2536 Å. predominating in the spectrum. A 15-cm. Petri dish containing 20 cc. of the bacterial suspension

was exposed at a distance of 50 cm. from the lamp and was agitated during exposure.

The survivor curves, $B(D)$, were obtained in a similar manner, except that immediately following exposure to the ultraviolet lamp the bacterial suspensions were exposed for one or two hours, at 8-inch distance, to the light of a 1000-watt projection lamp, and then plated on nutrient broth agar. The color temperature of the lamp was close to 3350°K. The bacterial suspensions were kept at 37°C. during exposure to the light of the projection lamp. In most of the series a 1-inch layer of a 0.05 molar solution of $CuCl_2$ was interposed between the lamp and the bacterial suspension.

As can be seen in figure 1, in semilogarithmic plot, both survivor curves A and B drop with increasing ultra-violet dose, D, slowly at first, then faster, and finally go over into a straight line.

As figure 1 shows, light-reactivation is a striking phenomenon. For an ultra-violet dose of $D = 190$ sec., for instance, the number of survivors falls in the absence of light-reactivation (curve A) from 10^8 to 700. With light reactivation, however, (curve B) the number of survivors only falls from 10^8 to 3×10^7; i.e., due to light-reactivation the titre of viable bacteria increases by a factor of about 40,000. In order to obtain the same drop in the survivors (from 10^8 to 3×10^7), in the absence of light-reactivation, a *lower* ultra-violet dose of $D = 75$ sec. would have to be employed. For every ultra-violet dose, D, which is followed by light-reactivation and thus leads to a certain number of survivors, $B(D)$, there can be found a lower ultra-violet dose, L, which in the absence of light-reactivation would lead to the same number of survivors; i.e., for which we have $B(D) = A(L)$.

Plotting L as a function of D in figure 1 we obtain for $L(D)$ a straight line going through the origin, for which we may write $L(D) = qD$, where q is a dose-independent constant smaller than 1. This means that there is a simple relationship between the survivor curves A and B which we may express by writing

$$B(D) = A(qD). \tag{1}$$

This relationship is confirmed by the nine series of experiments that were carried out. In any one of them the bacteria were light-reactivated by an exposure to the light of a projection lamp, at 8-inch distance, either of one hour or of two hours. After exposure of two hours the number of survivors obtained is, within the limits of experimental error, indistinguishable from the maximum reactivation that can be obtained with longer exposures to light. After exposure of one hour the number of survivors is lower, but the difference is not great. The q values obtained in these experiments vary slightly from series to series within the range of $0.32 < q < 0.42$. In the experiments shown in figure 1 we have $q = 0.4$.

If the light intensity used for light-reactivation is doubled by placing

the bacterial suspension closer to the projection lamp, the maximum number of survivors obtainable remains the same, only it is obtained with a shorter exposure to the light.

From the relationship (1) it follows that if we extrapolate in the semi-logarithmic plot the straight line portions of the survivor curves A and B to zero ultra-violet dose, we must be led back to the *same point.* This is shown by the dotted straight lines in figure 1.

In order to interpret our results we assume for the *sake of argument* that when bacteria are irradiated with ultra-violet light a "poisonous" chemical compound, P, is produced in an amount which is *proportionate* to the ultra-violet dose, D. We assume that this "poisonous" compound is produced in two forms: a form P_x, which is not sensitive to light and which is produced in the amount x_0, and a form P_y, which can be destroyed by light and which is produced in the amount y_0, so that $D = x_0 + y_0$. We further assume that the ratio x_0/y_0 is independent of the ultra-violet dose D. And finally we assume that the number of survivors after exposure to ultra-violet irradiation, with or without subsequent light-reactivation, is determined by the amount of "poison" that is present in the bacteria *at the time* they are incubated with nutrient medium and permitted to multiply.

On the basis of these assumptions, we may now account for the relationship (1) between the survivor curves A and B by saying that if we follow up the ultra-violet irradiation of a bacterial suspension by exposure to strong light for one or two hours we destroy all the poison present in the form P_y, and leave only the poison P_x, and by further saying that the amount of the poison present in the form P_x is given by $x_0 = qD$, where q is a dose-independent constant. The amount of "poison" left in the bacteria after light-reactivation is then given by qD, and therefore the number of survivors after light-reactivation, $B(D)$, can be taken from the survivor curve obtained in the absence of light-reactivation, $A(D)$, by writing $B(D) = A(qD)$.

If we now further assume that the light-sensitive variety of the poison, P_y, which is present in an amount y, is destroyed by light at a rate which is proportionate to its amount, y, we may write $\frac{dy}{dt} = -\alpha y$, where α is a function of the light intensity and independent of the dose, D. From this assumption it follows that if a bacterial suspension is exposed to an ultra-violet dose, D, and immediately afterward is exposed to strong light (say, to the light of a 1000-watt projection lamp at 8-inch distance) for a period of time, t, the total amount of poison, $L = x + y$, present in the bacteria is at any time, t, given by

$$L = x_0 + (D - x_0)e^{-\alpha t}. \qquad (2)$$

For an infinitely long exposure to light, we have $L_\infty = x_0$. We may then

write

$$\ln(L-L_\infty) = \ln(D-L_\infty) - \alpha t. \qquad (3)$$

The result of an experiment undertaken to check this relationship (3) is shown by curve I in figure 2. In this experiment we first exposed the bacterial suspension to the ultra-violet lamp for $D = 250$ sec. and then to the light of a 1000-watt projection lamp at 8-inch distance. We determined the number of survivors as a function of t, the time of exposure to the projection lamp, by plating on broth agar from aliquots taken from the bacterial suspension at different times, t, up to a time of 2 hours. From the number of survivors, we computed by means of the survivor curve, $A(L)$, the corresponding L values. As L_∞ we took the L value corresponding to the number of survivors obtained after 2 hours of exposure to the projection lamp.

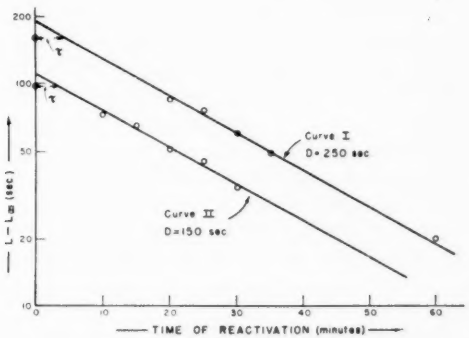

FIGURE 2

Experiment of June 23, 1949. Curve I gives in semi-log plot $L - L_\infty$ as a function of time of reactivation at 8 inches from 1000-watt projection lamp for bacteria that had received an ultra-violet dose, D, of 250 sec. Curve II is the same for bacteria that had received an ultra-violet dose of 150 sec. The interval τ represents the latent period of the process of reactivation.

As can be seen from curve I in figure 2 the points obtained fall on a straight line in accordance with equation (3). This type of relationship was confirmed in five series of experiments, giving values of α ranging from 2.19 to 2.38 per hour.

It should be noted however that the straight line I in figure 2 does not extrapolate back to the experimental point for zero exposure to light and the discrepancy corresponds to a latent period, τ, of about 3 minutes.

Line II in figure 2 differs from line I only in so far as it was obtained with an exposure to the ultra-violet lamp of $D = 150$ seconds. The fact that these two lines are parallel is in accord with the thesis that α is independent of the ultra-violet dose, D. The latent period τ manifested by line II is again about 3 minutes. Two series of experiments were performed to demonstrate in this manner the independence of α of the ultra-violet dose D.

Figure 3 shows the result of three experiments in which light-reactivation was carried out by exposing the ultra-violet irradiated bacterial suspension to the light of the projection lamp for $t = 20$, 25 and 30 min., respectively. From the number of survivors observed we computed, by means of the survivor curve, $A(D)$, the corresponding L values, and in each case we obtained for $L(D)$, as can be seen from figure 3, a straight line passing through the origin.

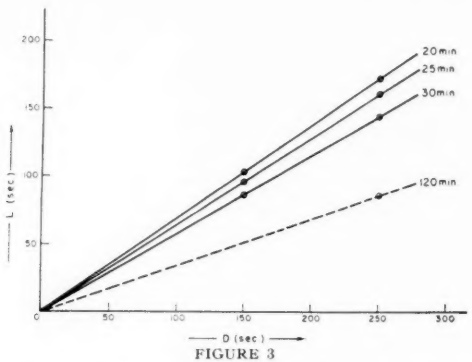

FIGURE 3

Experiment of June 23, 1949, gives L as a function of the ultra-violet dose, D, for 20, 25 and 30 minutes of light reactivation at 8 inches from 1000-watt projection lamp.

This result is to be expected provided that both α and τ are independent of the ultra-violet dose, D. Two such series of experiments were performed with identical results.

We were also interested to see in what manner the value of α depends on the intensity of the light which is used for reactivation. We varied this intensity by placing the bacterial suspension at different distances from the projection lamp. In such experiments we found that the value of α decreased with decreasing light intensity, but the decrease was less than strict proportionality with intensity would lead one to expect. The latent period τ increased in these experiments with decreasing light intensity.

We did not, however, in any of these experiments lower the light intensity very much below the value that obtains at 8inches from the pro-

jection lamp. We kept the intensity comparatively high in order to be able to neglect a reaction which takes place in the dark and therefore, of necessity, must also manifest itself if light-reactivation is carried out by means of very weak light. This dark reaction is easily observed if the bacterial suspension is irradiated with ultra-violet and then incubated in the dark at 37°C., for instance for 3 hours, prior to being reactivated at 8-inch distance from the projection lamp. The number of survivors after light-reactivation is then found to be much lower than is obtained when no dark incubation is interposed between ultra-violet inactivation and light-reactivation.

This concludes the report on our findings concerning the light-reactivation of ultra-violet inactivated bacteria in so far as these findings relate to the effect of light-reactivation on the number of survivors.

It is known that ultra-violet radiation not only kills bacteria but also produces mutants among the progeny of the survivors. We were interested to find out how light-reactivation affects the number of mutants which appear among these progeny. Particularly convenient for this purpose are mutants which manifest resistance to a bacteriophage, and we chose for the purpose of our investigation mutants resistant to the coli phages *T*4, *T*6 or *T*1.

M. Demerec[4] had studied the ultra-violet-induced mutations in coli to resistance to phage *T*1 and discovered that the great majority of the mutants do not appear immediately following ultra-violet irradiation, but that most of the bacteria have to go through several cell divisions before their mutation becomes phenotypically expressed.

A small minority of the mutants is phenotypically expressed prior to any cell division and A. Kelner finds that the appearance of such mutants is suppressed if the ultraviolet irradiation is followed by light-reactivation (see A. Kelner, abstracts of papers presented at the 49th General Meeting of the Society of American Bacteriologists, page 14, May 1948).

In our experiments we allowed, following irradiation, the survivors to go *through ten generations in liquid culture* and determined among the progeny the number of phage-resistant mutants per 10^8 bacteria.

If we are right in assuming that a poison is produced by the ultra-violet rays employed in our experiments, that the amount of this poison is reduced by light-reactivation, and that the amount of poison which is present in the bacteria when they are allowed to multiply determines the number of survivors, it then seems possible that this same "poison" might also determine the number of mutants—resistant to one of the phages—that appears among the progeny of the survivors. In this case we should expect that the number of mutants appearing among the progeny of the bacteria which were given a certain ultra-violet dose, *D*, and which were subse-

quently light-reactivated will be the same as the number of mutants appearing among the progeny of the bacteria which were exposed to a *lower* ultra-violet dose, qD, but which were *not* light-reactivated.

In our experiments the bacteria were exposed in saline suspension to different ultra-violet doses, D. Aliquots were then taken which were either incubated with lactate-ammonium phosphate medium at 37°C. under aeration or they were first exposed to the light of the projection lamp at 8-inch distance for one hour and were subsequently thus incubated. In either case the bacteria were allowed to go through about *ten generations* in liquid culture and were then assayed for mutants resistant to one of the coli phages $T4$, $T6$ or $T1$.

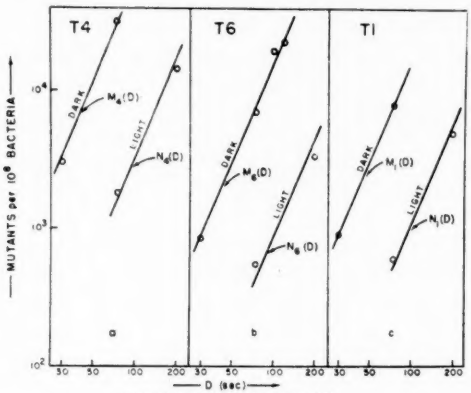

FIGURE 4 (*A*), (*B*) AND (*C*)

Experiment of July 2, 1949, gives in log-log plot the number of phage-resistant mutants per 10^8 bacteria as a function of the ultra-violet dose D both without (*DARK*) and with (*LIGHT*) light reactivation for phages $T4$, $T6$ and $T1$ in figures *a*, *b*, and *c*, respectively. The bacteria went through 10 generations in liquid culture prior to assaying for the mutants. The values shown in the graph are corrected for the spontaneous mutants which amounted to 800, 40 and 170 per 10^8 bacteria for $T4$, $T6$ and $T1$, respectively.

Figure 4 shows the result of one series of experiments in log log plot. The symbols $M_4(D)$, $M_6(D)$ and $M_1(D)$ relate to bacteria which were exposed to ultra-violet, were *not* light-reactivated, and went through ten generations in liquid culture (corresponding to an increase in bacterial titre by a factor of about 1000). Similarly $N_4(D)$, $N_6(D)$ and $N_1(D)$ relate to bacteria which were exposed to ultra-violet, were light-reactivated, and went through ten genera-

tions in liquid culture. These symbols designate the number of mutants resistant to phages $T4$, $T6$ and $T1$, respectively, which were found per 10^8 bacteria in the cultures—corrected for the spontaneous mutants that were obtained for zero ultra-violet dose—and plotted as a function of the ultra-violet dose, D.

Within the range of this experiment and within the limits of its accuracy, the lines $M(D)$ and $N(D)$ are represented in the log log plot by straight lines which are *all parallel* to each other. For each phage and for any given ultra-violet dose, D, the value of M is higher than the value of N. For every ultra-violet dose, D, which leads, when followed by light-reactivation, to a certain number of mutants, $N(D)$, there can be found a *lower* ultra-violet dose, L, which in the absence of light-reactivation leads to the same number of mutants; i.e., for which we have $N(D) = M(L)$.

Because the lines $M(D)$ and $N(D)$ are represented by *parallel* straight lines in the log log plot we may write:

$$N_4(D) = M_4(m_4D); \qquad N_6(D) = M_6(m_6D); \qquad N_1(D) = M_1(m_1D) \quad (4)$$

where m_4, m_6 and m_1 are dose-independent constants. The values of m_4, m_6 and m_1 taken from the experimental series plotted in figure 4 are 0.3, 0.3 and 0.32, respectively. For the same series of experiments the value of q was also determined and was found to be 0.35. Within the limits of our experimental accuracy we have thus found: $m_4 = m_6 = m_1 = q$. Two other series of experiments which were undertaken also gave, within the limits of experimental error, identical values for m and q.

It should be noted, however, that our experiments establish the validity of relationship (4) with much less accuracy than they establish the validity of relationship (1). In general, in our experiments the determination of the number of the mutants in any one single measurement was affected by an error of $\pm 50\%$, whereas the determination of the number of survivors in any one single measurement was affected only by an error of $\pm 20\%$. And more important, due to the shape of the survivor curve $A(D)$, a large percentage error in the number of survivors leads only to a small percentage error in the corresponding L values taken from the survivor curve A, whereas an error of 50% in the number of mutants, according to the curves M and N plotted in figure 4, leads to an error in the corresponding ultra-violet dose, D, of about 25%.

We have not investigated the ultra-violet-induced mutations to phage resistance with the same thoroughness as we have the effect of ultra-violet exposure on the number of survivors. Therefore at this time we can only say that our results to date are entirely consistent with the view that the effect of light-reactivation on the appearance of mutants among the progeny of the ultra-violet-irradiated bacteria is the same as is its effect on the number of survivors and that this effect consists in the reduction of the

effectiveness of the ultra-violet dose by the dose-independent factor q. This makes it possible to surmise that in our experiments the killing of the bacteria and the production of the mutants might be due to the *same* chemical effect produced by the ultra-violet irradiation.

[1] Kelner, A., PROC. NATL. ACAD. SCI., **35,** 73 (1949).
[2] Dulbecco, R., *Nature*, **163,** 949 (1949).
[3] Witkin, E. M., *Genetics*, **32,** 221 (1947).
[4] Demerec, M., PROC. NATL. ACAD. SCI., **32,** 36 (1946); Demerec, M., and Latarjet R., *Cold Spring Harbor Symposia*, **11,** 38 (1946).

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*SOME TWO-PERSON GAMES INVOLVING BLUFFING**

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1. Introduction.—It is now possible, with the aid of the von Neumann theory of games, to give a systematic theoretical treatment of the two-person zero-sum game. The techniques of this modern theory have been shown to have wide and important applications in such fields as economic theory, the theory of statistics and so on. Unfortunately, although the foundations of the theory are now classic, the actual solution of any particular game is generally a matter of some difficulty, involving a combination of frontal attack, applying routine methods, and a type of mathematical ingenuity that has been described as "low cunning."

Consequently, it is of interest to present a class of games, which may be handled by uniform techniques, in the hope that an accumulation of such examples will eventually render visible the mechanism behind the particular solutions, hitherto discovered only by artifice. Such has been the history of the theory of differential equations, the theory of probability and many of the other components of the mathematical edifice.

Consider the following two-person game where we call one player B, for bettor, and the other D, for dealer. Before play begins both players ante one. D then deals a card x_1 to B, where x_1 is a random number in the interval [0, 1] having the distribution function F_1, and a card y_1 to himself, y_1 also belonging to the interval [0, 1] with distribution function G_1. B initiates the betting, with the alternate possibilities of folding, in which case D wins the ante, or of betting an amount $f(x)$, $1 \leq f(x) \leq M$. Once B has bet, D has a choice of folding, in which case B wins the ante, of covering B's bet or of raising an amount $g(f(x), y)$, $1 \leq g \leq M$. If D raises, B has a choice of covering or raising and so on, for at most N_1 raises by either player.

After the initial betting, D deals two more cards, x_2 to B and y_2 to himself, and again B initiates the betting. This continues for at most N_2 draws. At the end of the betting, the hands are compared and the stronger hand wins the total amount wagered. The strengths of the hands are certain functions, $S(x_1, x_2, \ldots, x_n)$, $T(y_1, y_2, \ldots, y_n)$ of the cards, x_i, y_i. For the case of a deal and one draw, the above describes a poker game with two players; the case of several draws is analogous to Stud Poker; the case of several draws, but not simultaneously by both players, with one stage of betting and no raises, corresponds to Blackjack, sometimes called Twenty-One, with its arithmetically simpler cousin, Seven-and-a-Half; the case of no draws with the dealer always covering the bet is illustrated by Red Dog, and clearly many other common card games are described in many essentials by the above game.

Consequently, it is of some interest to investigate the problem of determining the best method of play for both players, where by "best" we mean the method that yields a maximum expectation.

The general problem seems quite difficult. In this paper we treat the simplified game where no draws are made after the initial deal, where there are only a finite number of bets allowed, $1 \leq z_1 < z_2 < \ldots < z_n$, with no raises, and where the distribution functions, F and G, are both equal to x.

However, it seems probable that the methods we present to treat the simplified version will be applicable to the N-draw game, and we hope to investigate the general question in the future.

That our procedures are applicable to the continuous case, and not to the discrete case, depends upon the result which is stated precisely below, that in certain continuous games only pure strategies are necessary, whereas the discrete game, in general, requires mixed strategies. Here the terms "pure" and "mixed" are used in the sense of game theory, and have no connection with the possible motives of either player. Given a card x, there are always several alternate procedures or strategies for either player to follow. A pure strategy is one which compels him to follow a unique course of action whenever he receives a card x; a mixed strategy is one which furnishes a rule for mixing the alternate available courses of action in certain proportions determined by x. A pure strategy then is a limiting form of a mixed strategy.

This result, that only pure strategies are required in the continuous case, seems quite unexpected, and at first sight to run counter to the known (heuristic) theory of bluffing. However, the paradox is easily resolved. In a continuous game, one allows the game, which furnishes a random card, to do the bluffing. It turns out that if the cards are dealt at random, any further randomization furnished by mixed strategies on the part of the players is superfluous.

This is not to say that there is no bluffing. Rather, one bluffs uniformly. This will be seen by examining the solution of the one-deal, no-draw game presented in the following paragraphs.

To the best of our knowledge, there have been only two previous mathematical discussions of the game of poker, the first by Borel,[1] and the second by von Neumann.[2] Borel's treatment depends more upon probabilistic considerations and involves no systematic use of game theory. One of the games discussed by von Neumann involves a choice of two bets for B, high or low (but no ante), and a choice for D, after having seen B's bet, of seeing or not. The game described in paragraph 3, following, differs from this only in allowing B the additional privilege of folding, at a cost, however. The solution obtained by von Neumann is, as in our case, a pure strategy for both players, with bluffing for B. Our methods, which lean heavily upon Theorem 1 below, are different from those of von Neumann.

2. *A Fundamental Theorem.*—The solution of a game is often simplified if it is known in advance that one of the players has a good pure strategy. Even if it is known in advance only that every mixed strategy for player I can be approximated by pure strategies, the value of the game can still be found by solving the restricted game in which both players are limited to pure strategies, but II discovers I's strategy. If in the restricted game I has a good strategy, this is also a good strategy in the original game.

There is a simple condition which sometimes ensures that mixed strategies may be approximated by pure strategies: if, at the beginning of the game, there is a chance move whose outcome is known to only one of the players, it may happen that he can use this chance move as a substitute for mixing strategies, so that a formally pure strategy, depending in an intricate way on the outcome of the chance move, can be constructed to approximate any given mixed strategy. This principle guarantees the existence of such approximating pure strategies in the one deal-no draw case of the game described above, and most probably in the general case too, although we have not as yet investigated this. That the actual good strategies are pure is not guaranteed by our principle, and, so far as we see, could not have been predicted in advance.

For our purpose, the relevant features of the game are the following, from I's point of view.

1. At the beginning of the game, there is a chance move consisting of the selection of a number x at random from the unit interval. x is known to I but not to II.

2. I's strategy is the choice of a number $i = 1, \ldots, N$, depending on x, i.e., a function $i(x)$, assuming only values $1, \ldots, N$.

3. The outcome $w(x, i; z)$, where z, II's strategy, may now vary over an arbitrary space, is for fixed i and z, a function of x consisting of R (fixed) continuous pieces, the modulus of continuity of the pieces being

uniform over (i, z). (In the poker game described, w consists of two constant pieces over the sets $x < y$, $x > y$.) Moreover, w is uniformly bounded; say $|w| \leq M$.

The payoff to player I, i.e., the expected outcome for fixed pure strategies of I and II, is then

$$v(i(x);\ z) = \int_0^1 w(x, i(x);\ z)dx. \tag{1}$$

THEOREM 1. *In a game satisfying 1, 2, 3, for every mixed strategy F for I and every $\epsilon > 0$, there is a pure strategy $i(x)$ such that, for every z,*

$$v(F;\ z) \leq v(i(x);\ z) + \epsilon. \tag{2}$$

Proof. A mixed strategy F for player I is a choice of N functions $p_1(x)$, $\ldots$, $p_n(x)$, $p_i(x) \geq 0$, $\sum_{i=1}^{N} p_i(x) = 1$ for each x, $p_i(x)$ specifying the probability he chooses i with a given x. Then $v(F;\ z) = \sum_{i=1}^{N} \int_0^1 w(x,\ i;\ z)\ p_i(x)dx$. Divide the interval $0 \leq \times \leq 1$ into disjunct subintervals I_1, $\ldots$, I_n, $m(I_j) < \delta$, where δ is chosen so that each continuous piece of each $w(x, i, z)$ varies by less than ϵ over any interval of length δ. Now divide I_j into N intervals I_{j1}, $\ldots$, I_{jN} so that

$$m(I_{jk}) = \int_{I_j} p_k(x)dx. \tag{3}$$

Then the pure strategy $i(x) = k$ on I_{jk}, $j = 1, \ldots, n$, $k = 1, \ldots, N$ is the required approximation to F. For

$$D = v(F;\ z) - v(i(x);\ z) = \sum_{j=1}^{n} \sum_{k=1}^{N} D_{jk}, \tag{4}$$

where

$$D_{jk} = \int_{I_j} w(x, k;\ z)p_k(x)dx - \int_{I_{jk}} w(x, k;\ z)dx.$$

Hence, $|D| \leq \sum_{j \in C} \sum_{k=1}^{N} |D_{jk}| + 2MNR\delta$, where C contains those j's for which every $w(x, k, z)$ for the given z is a single piece over I_j. The bound $2MNR\delta$ follows from the fact that there are at most NR j's not in C. For $j \in C$,

$$|D_{jk}| \leq |U_{jk} \int_{I_j} p_k(x)dx - L_{jk} \int_{I_{jk}} dx| \leq \epsilon\, m(I_{jk}), \tag{5}$$

where U, L are the upper and lower bounds of $w(x, k, z)$ over the interval I_j. Then

$$|D| \leq \sum_{j \in C} \epsilon\, m(I_j) + 2MNR\delta \leq \epsilon + 2MNR\delta. \tag{6}$$

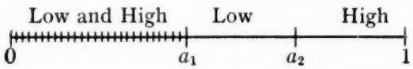
Since δ is arbitrarily small, the proof is complete.

3. *The Solution of a One-Draw Poker Game.*—In this section we present

the solution to the one-draw poker game with no raises and two bets of magnitude z_1, z_2, $z_2 \geq z_1 \geq z_0$, a constant specified below, the size of the ante being one. Complete details and discussion of the general case involving raises and a wider selection of wagers will be presented subsequently.

The solution shows that D's strategy is unique and involves no bluffing, in the sense that he never calls with a weak hand, while B's strategy is semi-determinate and involves one type of bluffing, betting high in low hands but not the other type, betting low on high hands.

B's strategy is shown diagrammatically by the following decomposition of the x interval [0, 1].



(1)

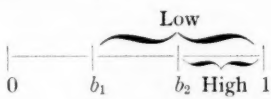
where

$$a_1 = 1 - c_1 \qquad (2)$$
$$a_2 = 1 - c_1c_2/2,$$

(where we have set $c_1 = 2/2 + z_1$, $c_2 = 2/2 + z_2$).

B bets high if $a_2 \leq x \leq 1$, low if $a_1 \leq x \leq a_2$ and if $0 \leq x \leq a_1$ must bet high H on a set of measure $(1 - c_2)c_1c_2/2$, low on a set L of measure $(1 - c_1)(c_1 - c_1c_2/2)$, and fold on the remaining part of $[0, a_1]$. The sets H and L are of fixed measure, but their location is arbitrary within $[0, a_1]$.

D's strategy is exhibited by



(3)

where $b_1 = a_1$, $b_2 = 1 - c_2$. D folds if $0 \leq y \leq b_1$, sees a low bet if $y \geq b_1$, and a high bet if $y \geq b_2$. From this it is clear why the exact location of the sets on which B bets high or low when he has a card in the interval $[0, a_1]$ is of no importance. The value of the game is

$$v = -(1 - c_1)^2 + c_1c_2(c_1 - c_2), \qquad (4)$$

which is always negative for $0 \leq c_2 \leq c_1 \leq c_0$, where c_0 is the smallest root of

$$c^3 - 8c^2 + 16c - 8 = 0, \qquad (5)$$

$c_0 \simeq 0.76$. The constant z_0 mentioned above is $2\left(\frac{1 - c_0}{c_0}\right) \simeq 0.62$. Thus, we see the character of the solution changes at the point where the game becomes fair to B. We have not as yet investigated the game in the

case $z_1 < z_0$, since the most interesting case is $z_2 > z_1 \geq 1$ (the amount of the ante).

* The results contained in this paper were obtained in the course of research conducted for The Rand Corporation, Santa Monica, California.

[1] Borel, E., and collaborators, *Traite du Calcul des Probabilités et de ses Applications*, Vol. IV, 2, Chapter V, Paris, 1938.

[2] von Neumann, J., and Morgenstern, O., *The Theory of Games and Economic Behavior*, Chapter 19, Princeton University Press, Princeton, 1947.

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*ON FLUCTUATIONS IN COIN-TOSSING**

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In the classical coin-tossing game we have a sequence of independent random variables X_v, $v = 1, 2, \ldots$ each taking the values ± 1 with probability $1/2$. We are interested in the signs of the partial sums $S_n = \sum_{v=1}^{n} X_v$. To eliminate the zeros of S_n we make the following convention: S_n is "positive" if $S_n > 0$ or if $S_n = 0$ but $S_{n-1} > 0$; otherwise S_n is "negative." The elegance of the results to be announced depends on this convention. Let N_n denote the number of "positive" terms among $S_1, \ldots, S_n$. We shall confine ourselves to an even n, noting only that $0 \leq N_{n+1} - N_n \leq 1$. In the following r and m are positive integers and $P(B|A)$ is the conditional probability of B under the hypothesis A.

THEOREM 1.

$$P(N_{2n} = 2r) = \frac{1}{2^{2n}} \binom{2r}{r} \binom{2n-2r}{n-r}.$$

THEOREM 2A.

$$P(N_{2n} = 2r | S_{2n} = 0) = \frac{1}{n+1}.$$

THEOREM 2.

$$P(N_{2n} = 2r | S_{2n} = 2m) = m \binom{2n}{n-m}^{-1} \sum_{i=m}^{r} \binom{2i}{i-m} \binom{2n-2i}{n-i} \frac{1}{i(n-i+1)}.$$

From these theorems it is easy to derive the following *limiting forms*

$$\lim_{n \to \infty} P(N_n \leq \alpha n) = \frac{2}{\pi} \arcsin \alpha^{1/2} \qquad 0 < \alpha < 1 \qquad (a)$$

(with the corresponding density $\pi^{-1}(\alpha - \alpha^2)^{-1/2}$), and

$$\lim_{n \to \infty} P(N_n \leq \alpha n | S_n = \lambda n^{1/2}) = \pi^{1/2} \int_{(1-\alpha)\lambda^2/\alpha} (\lambda^2(\alpha - 1) + \alpha y) y^{-3/2} e^{-y} \, dy \quad (b)$$

for $0 < \alpha < 1$ and for every fixed λ such that $\lambda n^{1/2}$ is a possible value of S_n. As $\lambda \to 0$ the last integral tends to α and for $\lambda = 0$ we get in the limit the uniform distribution as suggested by Theorem 2A.

The limiting form (a) of Theorem 1 is contained in a result of Erdös and Kac.[1] An analog to Theorem 1 for continuous random variables was proved by E. S. Andersen;[2] however, his result does not hold for arbitrary discrete variables, and Theorem 1 is not contained in Andersen's result.

The most surprising information is contained in Theorem 2A. To see this, note that the distribution of Theorem 1 departs radically from the familiar bell-shaped pattern. The limiting probability density curve resulting from Theorem 1 is U-shaped with the mean at the minimum. More precisely, while $\lim_{n \to \infty} E(N_{2n}/2n) = \frac{1}{2}$ this is the *least* probable value of $N_{2n}/2n$. This means that although the game is a symmetrical one (hence "fair") it is nevertheless more likely that one party "leads" in an overwhelmingly large proportion of the time than that each party leads about half of the time. In Theorem 2A it is plausible that the knowledge that S_{2n} vanishes should reduce the probability of extreme values of N_{2n}. It is noteworthy that this is true to the extent of a *uniform* distribution: at a moment when there is a tie all possible guesses about the fraction of time during which one partly has been leading are equally probable. This result contrasts not only with Theorem 1 but even more with a result of Paul Lévy[3] (Corollary 2, pp. 303–304). According to Lévy, if the condition $S_{2n} = 0$ is replaced by the hypothesis that at the $2n$th trial S_{2n} vanishes for the kth time, the limiting distribution as $k \to \infty$ of Theorem 2A is again the arc sin law (a) and no longer (b).

These results should serve as a warning to statisticians who might assume that fluctuation phenomena always follow the bell-shaped pattern and who would easily discover secular trends. If a coin is tossed once a second for a total of 365 days, the probability that one of the players will lead for more than 364 days and 10 hours is about 0.05! However, if it is known that the game concluded at a moment where neither player had a gain or loss, then the probability of such an extended lead is less than 0.0002.

Proof of Theorem 1. Let

$$p_{2r}(2n) = P(N_{2n} = 2r) \qquad \text{for } n > 0$$

$$p_0(0) = 1, \qquad p_r(0) = 0 \qquad \text{for } r > 0.$$

$$f_{2k} = P(S_j \neq 0 \text{ for } 1 \leq j < 2k;\ S_{2k} = 0) \qquad \text{for } k \leq 1;\ f_0 = 0$$

$$F(t) = \sum_{k=0}^{\infty} f_{2k} t^{2k} = 1 - (1 - t^2)^{1/2}.$$

Unless $r = 0$ or $r = n$, there is a smallest k such that $1 \leq k < n$ and $S_{2k} = 0$. All S_j with $1 \leq j \leq 2k$, are either all positive or all negative. These considerations lead to the recurrence relation

$$p_{2r}(2n) = \frac{1}{2} \sum_{k=0}^{n} f_{2k} p_{2r-2k}(2n - 2k) + \frac{1}{2} \sum_{k=0}^{n} f_{2k} p_{2r}(2n - 2k). \quad (1)$$

For $r = n$ we must add the probability that $S_{2k} > 0$ for $1 \leq k \leq 2n$, which is equal to

$$\frac{1}{2} \sum_{k=n+1}^{\infty} f_{2k} = \frac{1}{2} \binom{2n}{n} \frac{1}{2^{2n}}. \quad (2)$$

For $r = 0$ we must add the same quantity which now represents the probability that $S_{2k} < 0$ for $k \leq 2n$.

Introducing the generating function

$$g_{2n}(s) = \sum_{r=0}^{2n} p_{2r}(2n) s^{2r},$$

we obtain from formula (1)

$$g_{2n}(s) = \frac{1}{2} \sum_{k=0}^{n} f_{2k} s^{2k} g_{2n-2k}(s) + \frac{1}{2} \sum_{k=0}^{n} f_{2k} g_{2n-2k}(s) + \frac{1 + s^{2n}}{2} \binom{2n}{n} \frac{1}{2^{2n}}. \quad (3)$$

We now introduce the double generating function

$$G_s(t) = \sum_{i=0}^{\infty} g_{2i}(S) t^{2i}.$$

Then from formula (3),

$$G_s(t) = \frac{1}{2}\{G_s(t)F(st) + G_s(t)F(t)\} + \frac{1}{2}\{(1 - t)^{-1/2} + (1 - st)^{-1/2}\}, \quad (4)$$

where $F(t) = 1 - (1 - t^2)^{1/2}$, as implied by formula (2). Solving for $G_s(t)$ we get

$$G_s(t) = \frac{(1 - t)^{-1/2} + (1 - st)^{-1/2}}{(1 - t)^{1/2} + (1 - st)^{1/2}} = \frac{1}{(1 - t)^{1/2}(1 - st)^{1/2}}.$$

The coefficient of $s^{2r}t^{2n}$ is $p_{2r}(2n)$ and is that given in Theorem 1.

Proof of Theorem 2. We use the same notations as in the preceding proof except that now

$$p_{2r}(2n) = P(N_{2n} = 2r;\ S_{2n} = 2m).$$

The formula (1) remains valid except that for $r = n$ we must now add

$$W_n = P(S_k > 0 \text{ for } 0 < k \leq n;\ S_{2n} = 2m) = \frac{m}{n}\binom{2n}{n-m}\frac{1}{2^{2n}}$$

by a well-known formula (see reference 4, p. 153). For $r = 0$, formula (1) is valid as it stands with both sides vanishing. Using the generating function

$$W(s) = \sum_{n=m}^{\infty} w_{2n}s^{2n} = \left[\frac{1 - (1 - s^2)^{1/2}}{s}\right]^m$$

we obtain, instead of formula (4),

$$G_s(t) = {}^1/_2\{G_s(t)F(st) + G_s(t)F(t)\} + W(st).$$

Hence

$$G_s(t) = \frac{2W(st)}{(1 - t^2)^{1/2} + (1 - s^2t^2)^{1/2}} = 2W(st)\,\frac{(1 - s^2t^2)^{1/2} - (1 - t^2)^{1/2}}{t^2(1 - s^2)}. \tag{5}$$

For $m = 0$, $W(s) \equiv 1$. The coefficient of $s^{2r}t^{2n}(r \leq n)$ in the right-hand side of formula (5) is that of $s^{2r}t^{2n+2}$ in

$$-2(1 - t^2)^{1/2}(1 - s^2)^{-1}.$$

Thus it is the same for all r, $0 \leq r \leq n$. This means that $p_{2r}(2n)$, and consequently also $P(N_{2n} = 2r \mid S_{2n} = 0)$, is the same for all r, $0 \leq r \leq n$. Hence it is equal to $\dfrac{1}{n+1}$. Theorem 2A is proved.

If $m > 0$, the same coefficient is easily seen to be

$$\sum_{i=m}^{r} \left|2\binom{1/2}{n-i+1}\right| w_{2i}.$$

This is equivalent to the formula given in Theorem 2.

* This paper is connected with work under an ONR contract for research in probability theory at Cornell University.

[1] Erdös, P., and Kac, M., "On the Number of Positive Sums of Independent Random Variables," *Bull. Am. Math. Soc.*, **53**, 1011–1020 (1947).

[2] Andersen, E. S., "On the Number of Positive Sums of Random Variables," *Skand. Aktuarietid*, 1950, to appear.

[3] Lévy, P., "Sur certains processus stochastiques homogènes," *Compositio Math.*, **7**, 283–339 (1939).

[4] Uspensky, V., *Introduction to Mathematical Probability*, McGraw-Hill, New York, 1937.

A GENERALIZATION OF THE SCHWARZ-CHRISTOFFEL TRANSFORMATION*

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The classical Schwarz-Christoffel transformation provides a formula for the conformal mapping of the half-plane onto a plane polygon. A generalization enlarging the class of image domains to include many-sheeted plane polygons with interior winding points was known already to at least Schwarz and Schläfli.[1] This note is concerned with a further simple extension of the formula which delivers a useful class of mappings defined by multiple-valued functions. These mappings have been applied elsewhere by the author to certain hydrodynamical problems.[2]

Let $w(z)$ be a multiple-valued analytic function defined on the half-plane, $Im\ z > 0$. It is assumed that any regular function element of $w(z)$ can be continued along arbitrary paths to all but a finite number of points. It will be convenient in what follows to consider the image of a single-valued branch of $w(z)$ obtained by making a suitable cut or cuts in the half-plane which connect all the branch points of $w(z)$ with the boundary. If, on the image Riemann surface thus defined over the w-plane, the images of opposite points on the two edges of the cut are identified, we obtain an *ideal Riemann surface* or *Riemannian manifold* in the sense of Koebe.[3] (The image of a branch point, if it exists, is included as interior point of the surface.) This domain is a single-valued image of the half-plane under the mapping defined by (a branch of) $w(z)$. In the following we consider the conformal mapping of the half-plane onto certain Riemannian manifolds with polygonal boundaries. We have first,

THEOREM 1. *Let $w(z)$ be a possibly multiple-valued analytic function defined on the half-plane, $Im\ z > 0$. Let the function elements for $w(z)$ be such that in the neighborhood of any point $z = a$,*

$$w'(z) = (z-a)^{\alpha} P(z-a),\ P(0) \neq 0, \tag{1}$$

where $P(z-a)$ is regular at $z = a$, and α is non-zero for at most a finite number of points, $z = a_j$. Let $w(z)$ map the half-plane onto a Riemannian manifold M with a closed polygonal boundary having interior angles $\beta_k\pi$ at vertices which are images by $w(z)$ of the points $z = b_k (b_k\ real)$. Then $w(z)$ is of the form,

$$w(z) = A \int^z \{\prod_j [(z - a_j)^{\alpha_j} (z - \bar{a}_j)^{\bar{\alpha}_j}] \prod_k (z - b_k)^{\beta_k - 1}\}\, dz + B, \tag{2}$$

where A and B are complex constants.

Proof: The proof follows basically that of the classical Schwarz-Christoffel formula. We observe first that any two function elements for $w(z)$ over

the same point are linearly related, by virtue of a standard argument based on equation (1). Therefore the image of the real axis under the mapping determined by any branch of $w(z)$ is polygonal, the vertices being images of the points $z = b_k$, and $\beta_k\pi$ the magnitude of the corresponding angles. Hence $w(z)$ (whatever the branch) can be continued analytically into the lower half plane by reflection across the real axis. On the real axis $w(z)$ satisfies equation (1), the exponent α being equal to $\beta_k - 1$ at $z = b_k$ since the straight angle at b_k maps into an angle of magnitude $\beta_k\pi$; α is zero elsewhere on the real axis. Consider now the function w''/w'. By virtue of equation (1) and the foregoing, this function must be regular in the finite plane except for simple poles of residue α_i, $\bar{\alpha}_i$, $\beta_k - 1$, at $z = a_i$, $\bar{a}_i$, b_k, respectively. At the point at infinity, which is mapped by $w(z)$ into a boundary point, w''/w' must be regular and also vanishes. It follows that w''/w' is single-valued in the full z-plane, and regular except for the above-mentioned simple poles. We therefore have,

$$\frac{w''}{w'} = \frac{\alpha_i}{z - a_i} + \frac{\bar{\alpha}_i}{z - \bar{a}_i} + \frac{\beta_k - 1}{z - b_k},$$

from which equation (2) is obtained by integration.

If the exponent α in equation (1) is zero for all interior points, then equation (2) reduces to the classical Schwarz-Christoffel formula. Under certain circumstances, for example, if all the non-zero exponents α_i are positive integers, the (single-valued) mapping in equation (2) sends the half-plane into a many-sheeted Riemann surface with polygonal boundary, the images of the a_i being winding points of the surface.

The most general algebraic and logarithmic singularities are obviously not included in equation (1), and whether a generalization of equation (2) can be obtained for functions having such singularities is an open question.

Any Riemannian manifold M which is image of the half-plane under the mapping, equation (2), evidently has a polygonal boundary, is simply connected and contains a finite number of algebraic winding points. In addition, the coördinating transformations (Koebe: Bezugstransformation) which identify arcs on M are linear. For, it follows from equation (2) that if C is any arc in the z-plane containing no branch points of $w(z)$ in its interior, and C^+, C^- two arcs in the w-plane which are images of opposite edges of C, then a linear transformation of the w-plane transforms C^- into C^+. The vertices of the coördinating transformations (namely, the images of the branch points), are finite in number.

Conversely, let M be a Riemannian manifold with the above properties. Then by the principal theorems on the conformal mapping of Riemannian manifolds,[3] there is a function $z(w)$ which gives a $1-1$ mapping of M onto the open half-plane, any pair of coördinated arcs on M mapping into the

same arc in the z-plane. The latter arcs, considered as cuts in the z-plane, define the branch of the inverse function $w(z)$ having M as image. Let $w_+(z)$, $w_-(z)$ be function elements for $w(z)$ on opposite sides of an arc C of such a cut, assuming for convenience that C contains no branch points of $w(z)$. Consider the analytic continuations of $w_+(z)$ and $w_-(z)$ across C; (that such continuation is always possible follows from the definition of Riemannian manifold). Then, since $w_+(z)$ and $w_-(z)$ are linearly related on C, this relation holds in a full neighborhood of C. It follows that the functions w_+''/w_+' and w_-''/w_-' are identical in this neighborhood, and, by a simple consideration, that w''/w' is single-valued in the entire half plane. Hence, by using the Laurent expansion for w''/w', the function elements for $w(z)$ are seen to satisfy at any point a.

$$w'(z) = (z-a)^{\alpha} P(z-a) \exp Q(1/z-a),$$

where $P(z-a)$, $Q(1/z-a)$ are, respectively, power series in $z-a$ and $1/(z-a)$. However, the exponent $Q(1/z-a)$ must be constant, for otherwise, $\lim_{z \to a} w(z)$ would not exist and define an interior point of M, as required. Thus, the function elements of $w(z)$ satisfy equation (1); and since the number of winding points and vertices (of coördinations) on M is by assumption finite, the conditions on $w(z)$ for theorem 1 apply. Therefore, we have,

THEOREM 2. *Let M be a simply connected Riemannian manifold over the w-plane with polygonal boundary. Let the coördinating transformations on M be linear, the number of vertices of the transformations being finite. All other points on M are to be regular except for a finite number of algebraic winding points. Then there is a function $w(z)$ which maps the half-plane, Im $z > 0$, onto M, where $w(z)$ is of the form,*

$$w(z) = A \int^z \{\prod_j [(z - a_j)^{\alpha_j} (z - \bar{a}_j)^{\bar{\alpha}_j}] \prod_k (z - b_k)^{\beta_k - 1}\} \, dz + B.$$

In this expression, $\beta_k \pi$ are the interior angles of the polygonal boundary of M, and A, B, a_j, b_k, α_j, are constants, with Im $a_j > 0$, b_k real.

The actual determination of the constants in equation (2) is a matter of great practical difficulty, as it is for the classical Schwarz-Christoffel formula. The preceding theorem establishes only the existence of appropriate constants so that a branch of the function defined by equation (2) gives the desired mapping. Since a transformation of the half-plane into itself preserves the mapping, three of the constants b_k may be preassigned, or one a_j and b_k.

* Prepared under Navy Contract No. N6onr-180, Task Order 5, with Indiana University.

[1] Schwarz, H. A., *Ges. Math. Abh.*, **II**, 77 (1890); Schläfli, L., *J. reine angew. Math.*, **78**, 63–80 (1874).

[2] Gilbarg, D., and Serrin, J., *Free Boundaries and Jets in the Theory of Cavitation*, forthcoming publication.

[3] Koebe, P., "Allgemeine Theorie der Riemannschen Mannigfaltigkeiten," *Acta Math.*, **50**, 27–157 (1927). See also Courant, R., *Dirichlet's Principle*, Interscience (to be published); this uses the terminology *Riemann domain*.

ON SOME GENERALIZATIONS OF THE CAUCHY-FRULLANI INTEGRAL*

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1. A beautiful result attributed often to Frullani is contained in the general formula

$$\int_0^\infty \frac{f(at) - f(bt)}{t}\,dt = (f(\infty) - f(0)) \log \frac{a}{b}\ (a, b > 0), \qquad (1)$$

where $f(0) = \lim_{x \downarrow 0} f(x)$, $f(\infty) = \lim_{x \to \infty} f(x)$ and $f(x)$ is assumed L-integrable over any interval $0 < A \leq x \leq B < \infty$.[1]

2. Suppose that the integral

$$\int_0^A \frac{f(t)}{t}\,dt \qquad (2)$$

exists for any $A > 0$. Then the above formula can be replaced by

$$\int_0^\infty \frac{f(at) - f(bt)}{t}\,dt = f(\infty) \log \frac{a}{b}\ (a, b > 0) \qquad (3)$$

if $f(\infty) = \lim_{x \to \infty} f(x)$ exists. It can be expected that this formula remains valid if $f(\infty)$ is replaced by appropriate *mean values*.

Suppose, for instance, that $f(x)$ is *periodic with period* p and the integral (2) exists for any $A > 0$. Then $f(\infty)$ can be replaced by $\frac{1}{p}\int_u^{u+p} f(x)dx$ and we get the following very useful formula

$$\int_0^\infty \frac{f(at) - f(bt)}{t}\,dt = \frac{1}{p}\int_0^{u+p} f(x)dx \log \frac{a}{b}. \qquad (4)$$

3. Thus we obtain from

$$\int_0^{\pi} |\tan x|^{\alpha} dx = \frac{\pi}{\cos \frac{\alpha\pi}{2}} \quad (0 < \alpha < 1),$$

the formula

$$\int_0^{\infty} (|\tan ax|^{\alpha} - |\tan bx|^{\alpha}) \frac{dx}{x} = \frac{1}{\cos \frac{\alpha\pi}{2}} \log \frac{a}{b} \quad (0 < \alpha < 1;\ a, b > 0). \tag{5}$$

Similarly, it follows from

$$\int_0^{\pi} \log |\cos x| dx = \pi \log {}^1/_2$$

that

$$\int_0^{\infty} \log \left| \frac{\cos ax}{\cos bx} \right| \frac{dx}{x} = \log {}^1/_2 \log \frac{a}{b} \ (a, b > 0). \tag{6}$$

4. The condition of the periodicity of $f(t)$ can be replaced by an essentially more general one, that of the existence of the mean value

$$M(f) = \lim_{x \to \infty} \frac{1}{x} \int_0^x f(t) dt \tag{7}$$

and we obtain the following general theorem:

If the integral (2) exists for any $A > 0$ and the mean value (7) exists, we have for all positive a and b

$$\int_0^{\infty} \frac{f(at) - f(bt)}{t} dt = M(f) \log \frac{a}{b}. \tag{8}$$

5. To prove (8) we use the fact that

$$F(x) = \frac{1}{x} \int_0^x f(t) dt \qquad (x > 0)$$

is continuous for all $x > 0$ and tends to $M(f)$ as $x \to \infty$. We have on integrating by parts

$$\int_{bA}^{aA} \frac{f(t)}{t} dt = \int_{bA}^{aA} \frac{(tF(t))'}{t} dt = F(aA) - F(bA) + \int_{bA}^{aA} \frac{F(t)}{t} dt = F(aA) - F(bA) + F(\zeta) \log \frac{a}{b}$$

where $bA < \zeta < aA$. It follows

$$\lim_{A \to \infty} \int_{bA}^{aA} \frac{f(t)}{t} dt = M(f) \log \frac{a}{b}.$$

On the other hand we have

$$\int_0^{aA} \frac{f(t)}{t}\,dt - \int_0^{bA} \frac{f(t)}{t}\,dt = \int_0^{A} \frac{f(at)}{t}dt - \int_0^{A} \frac{f(bt)}{t}\,dt = \int_0^{A} \frac{f(at) - f(bt)}{t}dt$$

and our theorem is proved.

6. The assumption of the existence of (2) can be decomposed into two parts: (1) $f(t)$ is L-integrable over any interval $0 < \epsilon \leq t \leq A < \infty$; (2) $\frac{f(t)}{t}$ is L-integrable over any interval $0 \leq t \leq \epsilon$. The second part can be replaced by the assumption that $\lim_{\epsilon \downarrow 0} \int_{\epsilon}^{A} \frac{f(t)}{t}\,dt$ exists or by the hypothesis that $f(0) = \lim_{\epsilon \downarrow 0} f(t)$ exists and has the value 0. Both hypotheses are only special cases of a more general one which is obtained from the assumption about the behavior of $f(t)$ at $t = \infty$ by the transformation $t = \frac{1}{\tau}$. We come thus to the assumption

$$\epsilon \int_{\epsilon}^{1} \frac{f(t)}{t^2}\,dt \to 0 \qquad (\epsilon \downarrow 0).$$

More generally if we assume that together with (7)

$$m(f) = \lim_{\epsilon \downarrow 0} \epsilon \int_{\epsilon}^{1} \frac{f(t)}{t^2}\,dt \tag{9}$$

exists, we have

$$\int_0^{\infty} \frac{f(at) - f(bt)}{t}\,dt = (M(f) - m(f)) \log \frac{a}{b}. \tag{10}$$

7. The result obtained in (10) is in a certain sense the best result obtainable. It can be shown that if the integral at the left in (10) exists for some pairs of positive values a, b such that $\frac{b}{a}$ runs through a set of positive measure, then both limits $M(f)$, $m(f)$ exist and we have (10). However, the proof of this result is difficult and will be given in another publication.

Since finding the formula (10) I have discovered that the problem of convergence of the integral at the left in (10) has already been investigated and solved by K. S. K. Iyengar.[7] The necessary and sufficient conditions given by Iyengar consist in the existence of the four following limits:

$$\lim_{x \to \infty} \int_1^{x} \frac{f(t)}{t^2}dt, \qquad \lim_{x \to \infty} x \int_x^{\infty} \frac{f(t)}{t^2}\,dt \tag{11}$$

$$\lim_{x \downarrow 0} \int_x^1 f(t)dt, \qquad \lim_{x \downarrow 0} \frac{1}{x} \int_0^x f(t)dt. \tag{12}$$

It can be directly proved that both conditions (11) are equivalent to the existence of $M(f)$. Similarly, both conditions (12) are equivalent to the existence of $m(f)$. Iyengar's proof of his theorem and the simplified proof of it given by Agnew[2] are, however, still difficult since in both proofs certain theorems about non-uniform convergence are used which, although usually proved only in the case of convergent *sequences*, have to be used in the case of *continuous approximation*. Our proof of the necessity of the existence of $M(f)$ and $m(f)$ makes use of the theorem of Osgood, but exactly in the form proved by Osgood, that is for the case of *sequences* of functions.

8. Formula (1) can be considerably generalized by replacing *at* and *bt* by functions of t which to a certain extent are arbitrary. In this way we obtain a "three-function formula" giving the value of definite integrals containing three "arbitrary" functions.

This general formula cannot be directly reduced to (1) by substituting a new variable of integration. However, the interval of integration and the range of values of two of the functions can be reduced by such a transformation to the interval $(0, \infty)$. We obtain thus the canonical form of our formula

$$\int_0^\infty (\psi' g(\psi) - \varphi' g(\varphi))dx =$$

$$M(xg(x)) \log \left(\frac{\psi}{\varphi}\right)_{x=\infty} - m(xg(x)) \log \left(\frac{\psi}{\varphi}\right)_{x=0}. \tag{13}$$

The functions $\varphi(x)$ and $\psi(x)$ are supposed in (13) to be positive and absolutely continuous for $0 < x < \infty$ and to tend to 0 with $x \downarrow 0$ and to ∞ with $x \to \infty$. We assume further that with $x \downarrow 0$ and $x \to \infty$ the limits of $\dfrac{\varphi(x)}{\psi(x)}$ exist and are positive. The function $g(x)$ is assumed to be integrable over any closed interval of the positive x-axis and such that $M(xg(x))$ and $m(xg(x))$ exist.

9. Three special cases of (13) have been previously given. In 1823 Cauchy[3] gave the formula

$$\int_0^1 \left(\frac{\psi' f(\psi)}{1-\psi} - \frac{\varphi' f(\varphi)}{1-\varphi}\right) dx = f(1) \log \frac{\varphi'(1)}{\psi'(1)}. \tag{14}$$

Here $f(x)$ is continuous in the closed interval $< 0, 1 >$; φ and ψ are differentiable in $< 0, 1 >$ with positive derivatives and it is assumed that $\varphi(0) = \psi(0) = 0$, $\varphi(1) = \psi(1) = 1$. In 1841 Cauchy[4] published another formula

$$\int_0^\infty \left(\psi' \frac{f(\psi)}{\psi} - \varphi' \frac{f(\varphi)}{\varphi}\right) dx = f(0) \log \frac{\varphi'(0)}{\psi'(0)}. \qquad (15)$$

In this formula φ and ψ are positive in $(0, \infty)$ and have continuous derivatives which are positive at 0. Further we assume that $\varphi(0) = \psi(0) = 0$ and that as $x \to \infty$ $\varphi(x)$ and $\psi(x)$ tend to ∞. About $f(x)$ Cauchy assumes continuity for all $x \geq 0$. However, about the behavior of $f(x)$ for $x \to \infty$ Cauchy erroneously gives the condition $f(x) \to 0$. This condition is not sufficient for the validity of the above formula but Cauchy's argument remains valid if the existence of $\int_1^\infty \frac{f(x)}{x} dx$ is assumed.

Finally, 1891, M. Lerch[6] gave the formula

$$\int_a^b (g(x) - \varphi' g(\varphi(x)))dx = ((x-a)g(x))_{x=a} \log \varphi'(a) - ((x-b)g(x))_{x=b} \log \varphi'(b) \qquad (16)$$

that is obtained from (13) by obvious specializations and transformations.

Some of the results given in this note have been discovered independently by Professor Tricomi of the California Institute of Technology in his work on the Bateman manuscript project and are going to be published in the *Bulletin of the American Mathematical Society*. Professor Tricomi discovered our formula (4); further, a case of our formula (8), namely the case in which

$$\int_0^x f(x)dx - xM(f)$$

is $O(1)$—while in our theorem it is assumed that this difference is $o(x)$; as to the three-function formula, he finds the special case in which $g(x)$ has finite limits at 0 and ∞, respectively, and $xg(x)$ is periodic.

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[1] This formula was first published by Cauchy in 1823,[3] and more completely 1827[3a] with a beautiful proof which is used as standard in all textbooks today. About 1829 Frullani[5] published the same formula and mentioned that he had communicated it to plana in 1821. However, Frullani's proof is completely illusory.

[2] Agnew, R. P., "Limits of Integrals," *Duke Math. J.*, **9**, 10–19 (1942).

[3] Cauchy, A., *J. École Polytech. (Paris)*, **12** (1823); *Oeuvres compl.* (2), **I**, 335–339.

[3a] Cauchy, A., *Exercises de Mathematiques*, 1827; *Oeuvres compl.* (2), **VII**, 157.

[4] Cauchy, A., *Exercises Analyse*, **2** (1841); *Oeuvres compl.* (2), **XII**, 416–417.

[5] Frullani, G., "Sopra Gli Integrali Definiti (Ricevuta adi 21 Novembre (1829)," *Memorie della Societa Italiana delle Socienze*, **20**, 448–467 (1828).

[6] Lerch, M., "Sur une extension de la formule de Frullani," *Verhandl. Prager Akad., math.-phys. Klasse* I_2, 123–131 (1891).

[7] Iyengar, K. S. K., "On Frullani Integrals," *J. Indian Math. Soc.* (2), **4**, 145–150 (1940); reprinted in *Proc. Cambridge Phil. Soc.*, **37**, 9–13 (1941).

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